# HISTONE MODIFICATION DURING THE INDUCTION OF TYROSINE TRANSAMINASE.

by

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University of Cape Town

"If you can look into the seeds of time
and say which grains will grow and which will not ...."

(William Shakespeare - Macbeth i.iii.)

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# 1. INTRODUCTION

"The induction phenomenon is a useful model for the study of mechanisms which regulate the synthesis of specific enzymes in mammalian tissue, as well as for the rôle of hormones in this process" (1). The *in vivo* administration of hormone initiates a chain of reactions which culminate in the increase of protein synthesis (2). Many hepatic enzymes are thus affected (3) resulting in an increase of their activity (1,4,5).

Studies of the giant chromosomes in the salivary glands of *Chironomus tentans* provided the first indications that the earliest intracellular effects of hormone administration are at the genetic level (290). Injection of the insect moulting hormone, ecdysone, into last instar larvae of *Chironomus tentans* results in puff formation at specific loci of the chromosomes shortly after hormone administration (291,292). Since puffs are local and reversible alterations of the chromosome, formed at sites which are active in the synthesis of informational RNA (137,293,294), their formation shows a change in activity of particular gene loci in response to ecdysone.

Alterations in the chromatin of various target tissues (6-8) from the inactive heterochromatic (9,10) to the active euchromatic (8,10,11) condition, and increases in their template capacity for the DNA dependent RNA polymerase reaction after hormone treatment (12-16) have since been described. In addition, the polymerase enzymes themselves are stimulated - both the nucleolar (17-21) $Mn^{++}$- dependent and the extra-nucleolar (22-26) $Mg^{++}$- dependent enzyme species, which catalyze the synthesis of DNA-like RNA (AU rich) (27) )(28) and ribosomal RNA (GC rich (27) ) (29-33,14) respectively.

A further effect of hormone administration is the alteration of the microstructures of histones by acetylation (34,35) phosphorylation (36-39) and methylation (35,41,88). These basic chromosomal proteins (42) are closely associated with DNA in the chromatin (43,58) and their possible rôle as genetic repressors has been extensively discussed (44-47). DNA complexed with histone is not effective as a template for the DNA dependent RNA polymerase catalyzed synthesis of RNA (transcription) (43,48-51), and histones inhibit this reaction *in vitro* (52,53).

Which histone fraction is the most effective inhibitor remains a controversial subject (53-56). Spelsberg et. al. (57) have described the interaction between the RNA polymerase enzyme and different histone fractions. In their opinion, the lysine-rich histones have their effect by direct template repression, while arginine-rich histones decrease transcription by inactivation of the enzyme itself (50,51). The interaction between histones and DNA is electrostatic in nature (42,52,59,104), and any modification of the microstructure of these proteins which affect their basicity, results in a change of their affinity for DNA (46). Depending on the type of modification, this may be followed by a partial (60) or total (61) release of histone rendering the genetic material available for transcription (46,62).

Acetylation and phosphorylation reactions cause a decrease in the positive charge on the histone molecules and hence their release (46,60-62); methylation, however, raises the positive charge and effects a stronger binding between histone and DNA (63). The enzymatic modifications (47,53) of these proteins lead to structural alterations on or near potential DNA binding sites (61,64-67). Transacetylases, which catalyse the transfer of the acetyl group of acetyl co-enzyme A (68,69) to the ε-amino groups of internal lysine residues (41,70) of histones have been isolated from the nuclei of various organisms (41,65,68,71-75). Protein kinases (76-80) have been found which phosphorylate all histone fractions (81-84) to varying extents (46,83,85) at the $NH_2$-terminal serine residue (36,61,81,86) and at serine and threonine residues in the -COOH-terminal region (61,87). Methylated lysine (88-90), histidine (88,91) and arginine (40) residues of histones have been identified; each amino acid residue appears to be methylated by a different enzyme (92-94) although S-adenosyl-methionine is the common methyl donor (95-97). Enzymes which specifically remove these groups from the modified residues have been isolated (98,99).

None of the above alterations involve de novo synthesis of histone (72,95,97) - the processes are insensitive to puromycin (97) and do not occur at the time of amino acid incorporation into new histone (100,101). Because of the uptake into histone and the turnover of these groups in vivo at specific times in the cell cycle (37,102,289) their effects on histone have been correlated with the transcriptional level control (43-45,48,103) of genome activity.

Histones complex with corticosteroid hormones *in vitro* (149-155) (the arginine-rich fractions being more effective than the lysine-rich fractions (149-151,156) ) and thereby alter the template capacity of the associated DNA (16,151,157, 158). Some workers have found *in vivo* injected labelled hydrocortisone bound to histone (16,151,156,158), others have not (149,159). Tsai and Hnilica (149) associate this *in vivo* binding with contaminating cytoplasmic material in the isolated histone preparations. Polyamines have been reported to affect the accumulation of newly synthesized RNA in whole cells and in nuclear and nucleolar preparations *in vitro* (160,287). The role of the polyamines in the stimulation of RNA synthesis has not been elucidated.

From the accumulated evidence concerning the hormone-mediated derepression of the genome, the primary action of the hormone is thought to be at the transcriptional level (161). One of the early responses to such stimulation is the increased synthesis of all types of RNA in the nucleus (162-164). The production of messenger RNA is suggested by the ensueing protein synthesis (32) which is sensitive to such DNA-binding drugs (165-168) as Actinomycin D (162,169,170). Sequentially, the precursor ribosomal RNA's (171-174,33) (which mature into the characteristic 30S and 18S ribosomal RNA's (164,174, 175) ) appear in advance of the transfer RNA's and the DNA-like species (15,176,177), suggesting an initial activation of the A-T rich regions of the genome and a slightly later derepression of the GC-rich regions.

The newly synthesized RNA's are transported to the site of protein synthesis in the cytoplasm (24,178-180). Hormones may be involved also in this phase of enzyme induction by facilitating the transport of RNA across the nuclear membrane (181,182,296).

Whichever the mechanism(s) involved, the observed effects of hormone in target tissues, are the increases of RNA synthesis followed by the increases in protein synthesis.

In this study an attempt has been made to obtain information on the timing of enzymatic histone modification in the course of events occurring as a response to hormone administration *in vivo*.

Alterations in the binding of histone to DNA have also been related to the interaction of non-histone chromosomal proteins (NHC proteins) both with histone (104) and DNA (35,105,106). These NHC proteins have been suggested as regulatory intermediates in the restriction of DNA template activity by histone (107-112,130). The binding of these acidic proteins (111,113,114) to DNA is both species (35,105,106) and organ (115,120,130) specific, and their complexes with histone render the latter inactive as inhibitors of RNA synthesis (109,110,121-124). The NHC proteins themselves have been shown by some authors to be ineffective in this repression (110,116,119). Other workers, however, have found them capable of inhibiting the RNA polymerase reaction to a limited extent (117,118). Johnson et. al. (125) have noted a restriction of DNA template activity early in sea urchin development before the first histones are synthesized.

Chromatin of a given organism, with a high template activity, contains more NHC proteins than does chromatin with low template activity (52,126-131). In response to hormone treatment (110,132-134) target cells accumulate both RNA and NHC proteins (135-139). In labelling experiments the specific activities of the latter are proportional to the synthesis of DNA-like RNA (140). The RNA synthesized using chromatin from different organs as a template shows organ specificity (108). The artificial reconstruction of such chromatin using DNA and NHC protein from different organs results in hybrid chromatins. If these hybrid chromatins are used as templates, the organ specificity of the RNA synthesized is determined by the parent organ of the NHC proteins (107,108,141).

Another candidate for conferring specificity of repression on histones is the chromosomal RNA of Bonner et. al. (142,143). Other workers have been unable to confirm Bonner's results and suggest that this species is a contaminant transfer RNA (144-146). Hormone induced modifications of the DNA molecule itself have been described (133,147,169) whereby its capacity for histone binding becomes reduced (79,148).

## 1. 2. PLAN OF RESEARCH

A severe lack of information exists concerning time correlation between gene activation, enzymatic histone modification, RNA synthesis and the ensueing protein synthesis, investigated in one experimental model. Many investigations have been reported combining any two of these four processes. The induction of enzyme activity by steroid (3,4,183-185) and polypeptide (2,4,186) hormones is well documented. That template activation and RNA synthesis precede protein synthesis is an established fact. Histone modification by acetylation (187-189) and phosphorylation (37,38) appears to occur in advance of template activation and RNA synthesis (190,65), while methylation appears to be a relatively late event (191,63). The general picture arising from the available information, gained from many different experimental systems, seems to show that derepression of the genome involves acetylation and phosphorylation of arginine-rich histones, and an elevated rate of RNA synthesis, followed by an increase in protein synthesis and methylation of most fractions of histone.

To verify this general tentative view on the process of derepression, a detailed study of the time course of these events under identical experimental conditions is necessary.

As inducers of enzyme activity, the hormones hydrocortisone and insulin were chosen. Both were known to increase the activity of hepatic tyrosine transaminase, L-tyrosine-2-oxoglutarate-aminotransferase EC 2.6.1.5 (TAT*) (2-4). This increase is due to increased de novo synthesis (1,4,5) and not to decreased degradation (192,193). The enhanced transaminase activity resulting from steroid or insulin stimulation can therefore serve as a satisfactory model to investigate the process of induction. The following parameters have been examined : time course of enzyme activity, of RNA synthesis, and of histone modification by phosphorylation, acetylation and methylation during induction with hydrocortisone and insulin.

* TAT - abbreviation Tyrosine aminotransferase.

## 2. MATERIALS

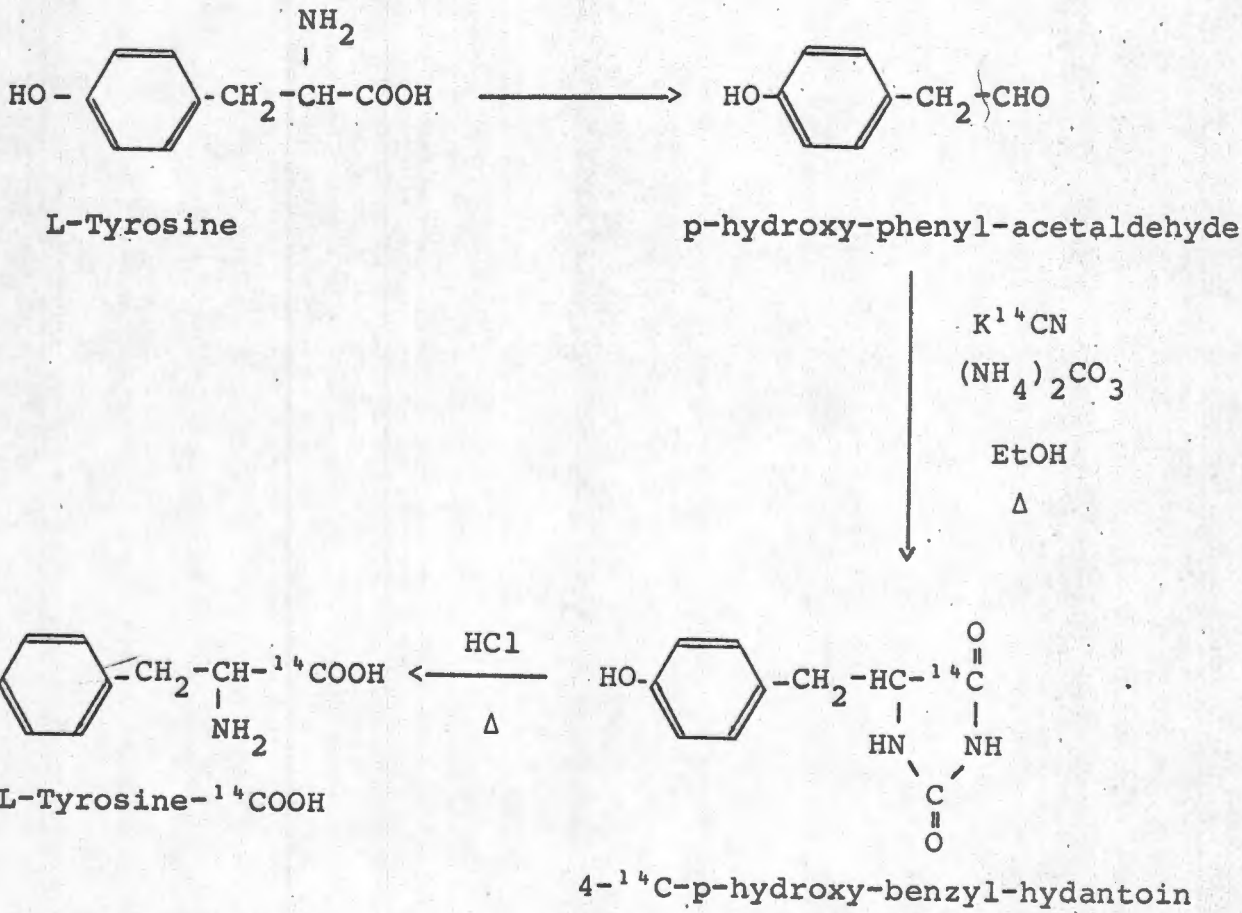
All chemicals used were of Analar grade or equivalent supplied by various companies.

Radioactive chemicals were obtained from the Radio-Chemical Centre, Amersham, England.

Tyrosine labelled in the $C_1$ position was not commercially available, and was therefore synthesized.

### 2.1. SYNTHESIS OF TYROSINE-$^{14}$COOH

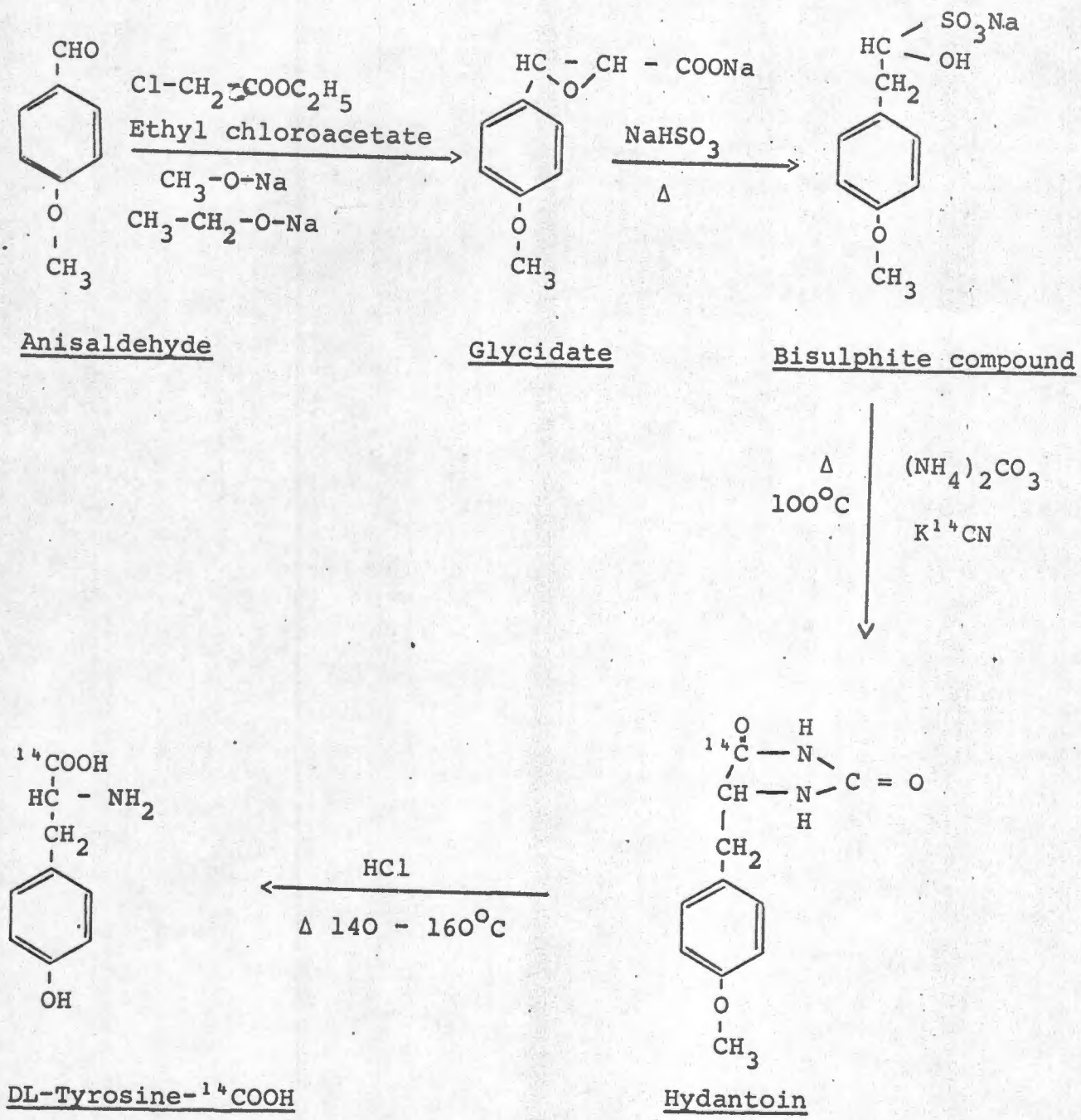
$C_1$ labelled amino acids can be synthesized according to the following reaction :




Various attempts to prepare the p-hydroxy-phenyl-acetaldehyde via oxidative decarboxylation and deamination of tyrosine with ninhydrin in order to prepare the 4-$^{14}$C-p-hydroxy-benzyl-hydantoin as intermediate for the synthesis of carboxyl labelled tyrosine failed.

Figure 2.1. is a schematic representation of the method finally employed (194).

Figure 2.1. SCHEMATIC REPRESENTATION OF THE METHOD USED FOR THE SYNTHESIS OF TYROSINE-$^{14}$COOH



2. 1.i. p-methoxy-phenyl-acetaldehyde sodium bisulphite compound :

7 g Sodium were dissolved in 50 ml absolute ethanol and 50 ml absolute methanol, and cooled.

The sodium-ethylate, sodium-methylate mixture was added to a solution of 32.0 ml ethylchloroacetate, 36.5 ml anisaldehyde in 70 ml anhydrous ether at a rate whereby, with salt-ice cooling and continuous sitrring, the temperature was maintained at 2°C. When all the sodium alcoholate had been added, cooling was discontinued, and the mixture allowed to reach room temperature. It was then poured into 650 ml cold distilled water. The aqueous solution was extracted 5 times with 100 ml portions of ether. The ether extract, washed with 300 ml cold water, 300 ml cold 3% $NaHCO_3$ and again 300 ml cold water, was chilled and poured with constant stirring into an ice-cold solution of 6.9 g sodium in 100 ml methanol and 6.0 ml water.

The sodium p-methoxy-phenyl-glycidate which precipitated from the mixture at 4°C within ten minutes was collected by filtration with suction. It was washed with 10 ml methanol - 50 ml ether, and 40 ml ethanol - 30 ml water, and then added to a vigorously stirred solution of 70 g $NaHSO_3$ in 250 ml boiling water. The pH was brought to about 4.5 by addition of 8 ml 100% acetic acid and the solution allowed slowly to cool to 5°C.

The resulting heavy precipitate of p-methoxy-phenyl-acetaldehyde sodium bisulphite compound was collected by centrifugation and stored until use at -14°C.

2. 1.ii. 5(p-methoxy-benzyl) Hydantoin-4-$^{14}C$

600 mg Bisulphite compound
600 mg $(NH_4)_2CO_3$ (powdered)
64 mg KCN
1 mg (0.6 mCi) $K^{14}CN$ (39.6 mCi/mM)
) 1 m mole
3.0 ml 50% EtOH-$H_2O$ (v/v)

were heated in a sealed ampoule for 4 hours at 100°C. After the tube had been cooled and opened, the mixture was gradually warmed to 104°C to decompose excess $(NH_4)_2CO_3$ and remove the ethanol. 3.0 ml hot water was added, and the mixture was transferred to a centrifuge tube and cooled for a minimum of 2 hours.

The hydantoin precipitate was collected by centrifugation, heated in 50% ethanol-water (v/v) until it dissolved, transferred to an ampoule and cooled to room temperature. It was then dried by gentle heating (50°C) under a stream of nitrogen.

2. 1.iii. $^{14}$C-Carboxyl-labelled DL-Tyrosine

The hydantoin was heated in the sealed ampoule with 3.0 ml 6 M distilled hydrochloric acid for 4 hours from 140°C to 160°C. The tube was cooled and opened. 3.0 ml water were added to the contents which was then heated in a water-bath and treated hot with charcoal. The solution was neutralised with ammonium hydroxide to pH 6 and transferred to a centrifuge tube for cooling.

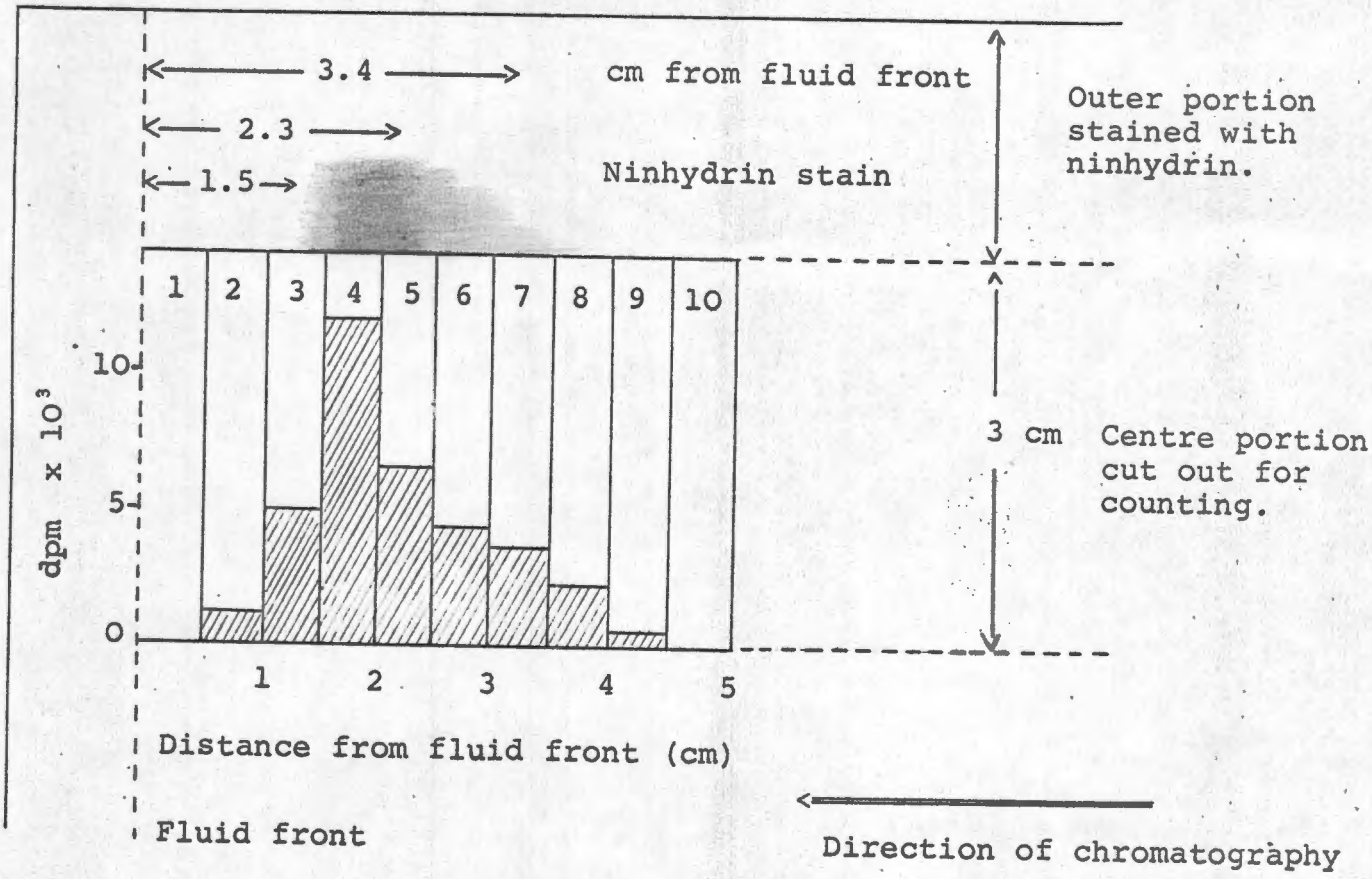
The resulting brownish precipitate was washed with cold water (until these washes no longer showed a positive reaction with Nessler's Reagent), cold ethanol and cold ether and finally dried over clacium chloride yielding white crystals of DL-tyrosine-$^{14}$COOH with a specific activity of 0.56 µCi/µmole. (Total yield based on K$^{14}$CN = 50%).

This radioactive DL-tyrosine had an UV absorption spectrum and molar extinction coefficient identical to commercial tyrosine, and the determination of α-amino groups by the ninhydrin method of Troll and Cannon (195) gave 97% the theoretical value.

Thin layer chromatography using 96% ethanol-water (7:3 v/v) as mobile phase yielded a single ninhydrin positive spot with the Rf value of tyrosine, and no additional organic components could be detected after exposure of the chromatogram to iodine vapour. The radioactivity was confined to the ninhydrin positive fraction, though large excesses of tyrosine had been applied to the chromatogram in order to detect contaminations (Figure 2.2.).

All radioactivity of a solution of the 1-$^{14}$C-tyrosine preparation was adsorbed by a Dowex 50(X4) $H^+$ column. Extensive washing of the column with water removed none of the adsorbed activity, whereas 1 N $NH_4OH$ released all the radioactivity.

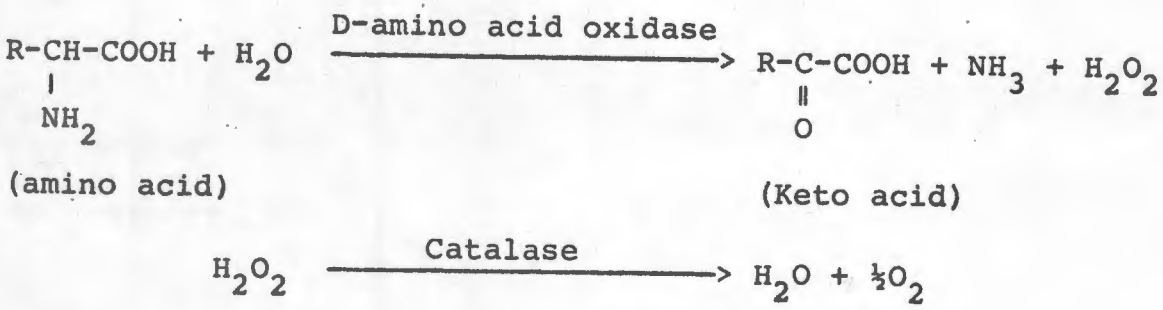
Figure 2.2. THIN LAYER CHROMATOGRAPHY OF DL-TYROSINE-$^{14}$COOH




Development with 96% ethanol-water (7:3 v/v), 60 minutes. A centre portion was cut out and divided into 0.5 cm strips, the radioactivities of which were determined. The remainder was stained with 0.1% Ninhydrin in acetone.

2. 1.iv. $^{14}$C-Carboxyl labelled L-Tyrosine

Pure L-tyrosine-$^{14}$COOH was prepared from the DL-tyrosine-$^{14}$COOH by enzymatic destruction of the D-isomer with D-amino acid oxidase (196) according to the following reaction :




The incubation mixture in a total of 34 ml, contained the following :

26.997 µmoles DL-tyrosine-$^{14}$COOH in phosphate buffer pH = 7.1
(= 13.267 µmoles D-isomer)

13.3 U D-amino acid oxidase (EC 1.4.3.3. Boehringer crystalline suspension in 1.8 M $(NH_4)_2SO_4$ solution, pH = 6.5; 5 mg/ml 15 U/mg).

19,500 U Catalase (EC 1.11.1.6 Boehringer crystalline suspension in thymol saturated water pH = 6.0; 20 mg/ml 39,000 U/mg).

5.0 ml 0.1 M Sodium pyrophosphate-hydrochloric acid buffer pH = 8.3.

The mixture was incubated for 4 hours at 37°C in a water-bath with continuous shaking, and oxygen passed through the solution through a sintered glass filter stick. Thereafter the contents of the incubation flask was freeze-dried, the resulting material taken up in 9 ml distilled water, and applied to a 10 ml column of Dowex 50 (X4) $H^+$.

The water eluent containing the keto acid arising from the D-isomer was collected until no further radioactivity emerged. The combined fractions yielded 40% of the total activity incubated with the oxidase enzyme. Amino acid was eluted with 2 N ammonium hydroxide (48% of total activity), the combined fractions freeze-dried and the dry material taken up in 20 ml pH 7.1 phosphate buffer.

The ratio of $^{14}$C keto acid/$^{14}$C amino acid indicated incomplete removal of the D-isomer. This solution was therefore re-treated with oxidase and subsequently processed under the same incubation conditions as described above.

A small amount (6%) was recovered as keto acid in the water eluent and 90% as amino acid in the ammonium hydroxide eluent. The latter was freeze-dried as the material still contained denatured enzyme protein, suspended in 3 ml distilled water, and the tyrosine dialysed out into 6 changes of 5 ml water.

This final L-tyrosine-$^{14}$COOH solution had a specific activity of 0.54 μCi/μmole.

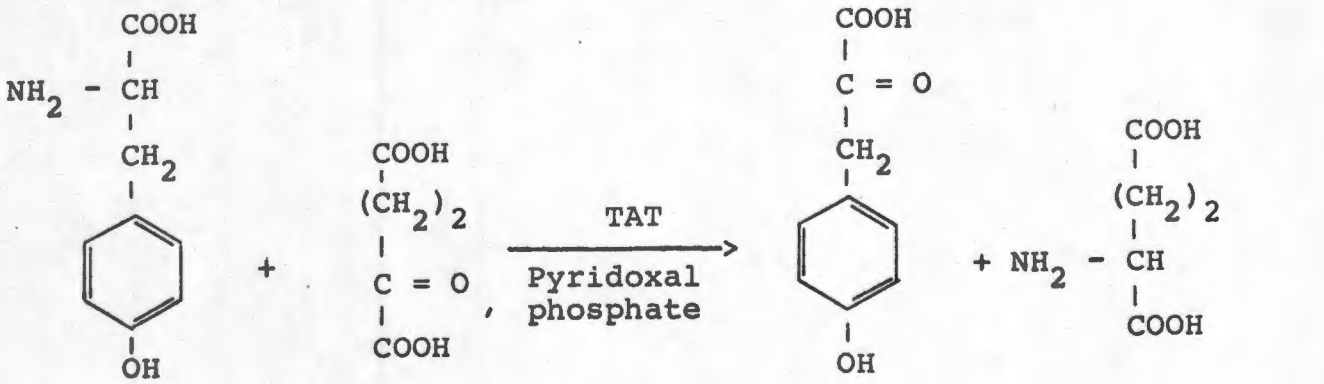
# 3. METHODS

## 3.1. TYROSINE TRANSAMINASE

### 3.1.1. Principles of TAT Assay

The transaminase reaction is the pyridoxal phosphate dependent transfer of the α-amino group of tyrosine from the amino acid to the C2 position of the acceptor molecule, α-keto glutaric acid. The co-enzyme reacts with tyrosine to form a Schiff base from which p-hydroxy-phenyl-pyruvic acid is released; the remaining pyridoxamine phosphate then reacts with α-keto glutaric acid to form a second Schiff base which hydrolyses yielding glutamic acid and the restored co-enzyme.

The overall reaction may be written :




Tyrosine | α-Keto glutaric acid | Keto acid (p-hydroxy-phenyl-pyruvic acid) | Glutamic acid

Assay methods for this enzyme would entail a) measurement of the keto acid formed, or b) measurement of the unreacted substrate remaining after the enzyme reaction.

a) The assay method of Tomkins et. al. (197) involves the alkali-catalyzed oxidation of p-hydroxy-phenyl-pyruvic acid by molecular oxygen to p-hydroxy-benzaldehyde and oxalate. The extinction at 331 mμ of p-hydroxy-benzaldehyde is taken as a measure of keto acid product formed. In our attempts to apply this assay, considerable difficulties were encountered, largely because of the limited stability of the aldehyde, and the difficulties to clearly identify spectrophotometrically the small amount of p-hydroxy-benzaldehyde in the presence of a large excess of tyrosine.

The method of McFarlane (198) for the assay of leucine transaminase is based on the decarboxylation of the 1-$^{14}$C-keto acid arising out of the transamination of 1-$^{14}$C labelled amino acid. The decarboxylation is achieved through the reaction of the keto acid with ceric sulphate ($Ce(SO_4)_2$), yeilding labelled $CO_2$ which is a measure of the keto acid formed during the transamination reaction. Attempts to apply this method for the assay of tyrosine transaminase were unsuccessful. Though the $Ce(SO_4)_2$ decarboxylation is considered to be specific for α-keto acids under the conditions applied (199) and has been shown to be α-keto acid specific in the leucine transaminase test, it was found that 1-$^{14}$C-tyrosine also underwent decarboxylation by $Ce(SO_4)_2$.

b) An alternative would have been to decarboxylate unreacted tyrosine after the enzyme reaction had been terminated, and, as above, to collect and count the $CO_2$ produced. However, attempts to decarboxylate unreacted tyrosine quantitatively with ninhydrin both in citric acid medium (200) and organic medium (195) also proved unsuccessful under the conditions required.

A new method of assay was thus developed based on the following principle ; unutilized substrate (α-amino acid) at the end of the reaction was bound onto Dowex (X4) $H^+$ cation exchange beads, the unbound radioactive keto acid removed by exhaustive washing with water, and the amino acid finally released from the ion exchange resin with ammonium hydroxide. The radioactivity of this solution was then determined. Enzyme activity (micromoles tyrosine consumed) corresponded to the difference between total $^{14}$C activity at zero time and ion exchanger-trapped radioactivity at the end of the experiment.

### 3. 1. 2. TAT Assay Method

#### (1) Preparation of Crude Extract (197)

After a standard period of 20 hours fasting, experimental animals were stunned by a blow on the head, decapitated and exsanguinated. The livers were rapidly exicsed, and homogenized in 3 x volume of ice-cold 0.15 M KCl containing $10^{-3}$ M EDTA adjusted to pH = 7.6 with sodium phosphate, in a glass homogenizer with teflon pestle. The homogenate was centrifuged at 20,000 x g for 30 minutes at 4°C in a Sorvall RC 2 centrifuge and the resulting sediment discarded. Aliquots of the supernatant were used as enzyme extract.

(2) Assay :

The incubation mixture contained

0.1 µmole pyridoxal phosphate
9.0 µmoles α-keto glutarate (di-sodium salt adjusted to pH = 7.6)
1.0 µmole EDTA
13.4 µmoles tri-ethanol-amine
0.0346 µmoles $^{14}$C-tyrosine (62 µg/ml aqueous solution adjusted to pH = 7.6 with di-sodium phosphate)
1.2 µmoles carrier L-tyrosine (dissolved in phosphate buffer pH = 7.6)

which, together with the enzyme extract, made up a total volume of 1.8 ml pH = 7.3. Addition of extract initiated the reaction and, in initial experiments, incubation continued with shaking for 15 minutes at 37°C. The reaction was at first terminated by heating the incubation mixture at 100°C in a boiling water-bath for 2 minutes. However, termination by addition of the ion exchanger resulting in an immediate drop of pH, proved a more reliable method. The solution was allowed to exchange with 500 mg Dowex $H^+$ beads for a standard period of 10 minutes after which time the supernatant fluid was drawn off with a finely-tipped Pasteur pipet. The beads were washed with 2 x 2.0 ml distilled $H_2O$, and the absorbed unreacted substrate released by elution with 2.0 ml 1 N ammonium hydroxide. 0.4 ml Aliquots of the $NH_4OH$ supernatant were taken for radioactivity determinations.

For the TAT reaction to proceed, α-keto glutaric acid is an essential component of the incubation mixture, whereas oxidative deamination can proceed in the absence of a keto acid acceptor. To exclude the presence of contaminating L-amino acid oxidase activity in the extract, experiments including and excluding α-keto-glutarate were carried out. In the absence of acceptor keto acid, 15 minutes incubation yielded 100% recovery of radioactivity as amino acid, indicating the absence of an oxidative deamination reaction.

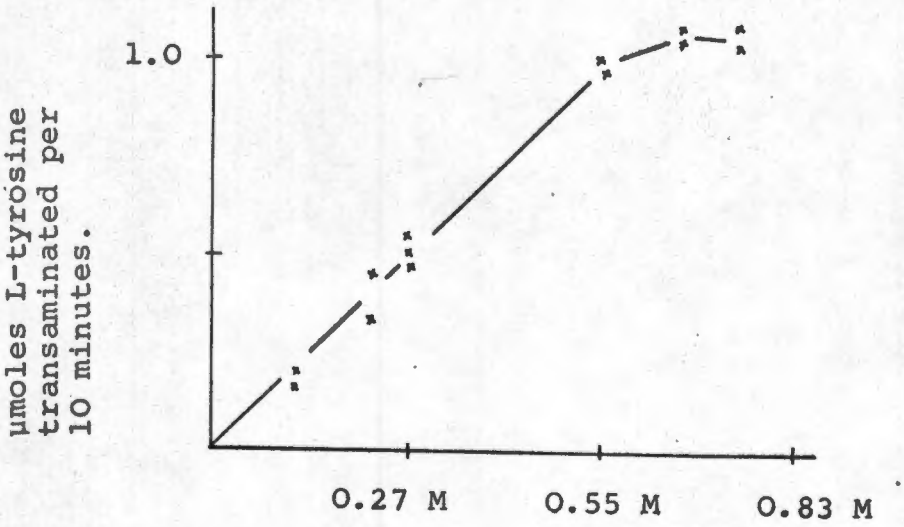
Under identical conditions, in the presence of $4.5 \times 10^{-3}$ M and $9 \times 10^{-3}$ M α-keto glutarate, 35% of the substrate was consumed. α-Keto glutarate was therefore limiting for the activity assayed, ruling out the possibility of contaminating amino acid oxidase.

L-tyrosine has a solubility of only 0.453 mg/g $H_2O$ at 25°C (201) or 2.5 µmoles/ml, just allowing substrate saturation conditions (Figure 3.1). However, the amount of tyrosine that could be included within the assay volume was seriously limited. This led to a rapid depletion of substrate unless the amount of enzyme was kept very low and the incubation temperature reduced from the initially used 37°C, to 25°C. The reaction rate was linearly dependent on the amount of enzyme extract at 10 minutes incubation, up to an extract volume of 0.2 ml (Figure 3.2). Attempts to increase the amount of substrate by saturating the assay volume with tyrosine led to uncontrollable inaccuracies. The TAT assay therefore has inherent limitations which may lead to an under-estimation of high enzyme activities.

After establishing these properties of the assay system, the following standard procedure was adopted :

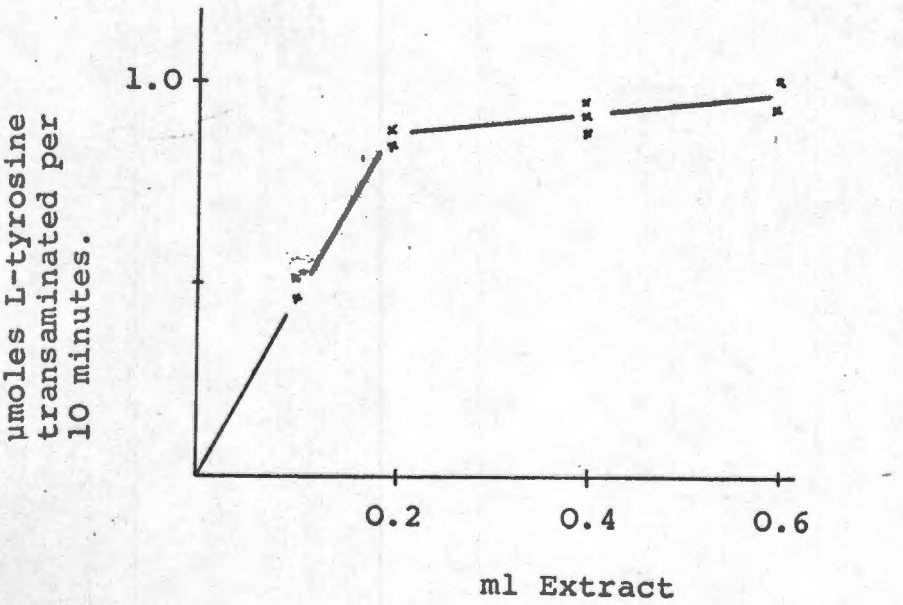
The incubation medium as described above, total volume of 1.775 ml pH = 7.6, and a tyrosine concentration of $0.7 \times 10^{-3}$ M (total activity 40,000 dpm $^{14}C$) was equilibrated to 25°C in a water-bath. Addition of 0.025 ml enzyme extract initiated the reaction, incubation was carried out with shaking at 25°C for 10 minutes, and the reaction stopped by addition of 500 mg Dowex $H^+$ beads to the incubation mixture. After 10 minutes mixing with a magnetic stirrer, the beads were allowed to settle, a further 500 mg Dowex beads were added and the mixing repeated. A standard period of 5 minutes was maintained to allow all the beads to settle before the supernatant fluid was drawn off, and the beads washed with 2 x 2.0 ml distilled $H_2O$. The absorbed tyrosine was eluted with 2.0 ml 1 N $NH_4OH$. Aliquots were counted in 10 ml of scintillation cocktail. TAT activity was expressed in µmoles tyrosine transaminated per mg protein per 10 minutes.

Figure 3. 1. Substrate dependence of TAT Assay




Incubation : 10 minutes at 25°C using 0.6 ml crude enzyme extract.

Figure 3. 2. Extract volume dependence of TAT assay




Incubation : 10 minutes at 25°C using 0.7 x $10^{-3}$ M substrate.

Protein was determined by a Biuret method (202); μmoles tyrosine transaminated were calculated from the equation :

$$\frac{\text{radioactive tyrosine added (dpm) - radioactive tyrosine recovered (dpm)}}{\text{specific activity of substrate (dpm/μmole)}}$$

Enzyme extracts were assayed immediately after preparation as freezing of the extract for storage and subsequent thawing resulted in variable loss of activity.

## 3. 2. ISOLATION OF RNA

### 3. 2. 1. ISOLATION PROCEDURE

Various methods were investigated :

i) The method of Kirby (203) for the preparation of total RNA from rat liver proved unsuitable for the purpose of this project. RNA samples prepared by this method, which involved sodium dodecyl sulphate treatment and hot phenol (60°C) extraction, sedimented on sucrose gradients (see 3.2.2) as a single band with an S value of approximately 10. Gel filtration on sephadex G25 also showed that this preparation consisted largely of, probably degraded, low molecular weight RNA.

ii) Ribosomal RNA was isolated by the method of Moldave et. al. (204). The crude ribosomes (205) were treated with sodium dodecyl sulphate, and phenol extraction performed at 4°C. The ethanol precipitate from the combined aqueous phases was dissolved in potassium acetate and the RNA reprecipitated with cold ethanol. Sucrose gradient centrifugation of these samples yielded the characteristic RNA peaks at 18S and 30S and, in addition, a minor low molecular weight peak at approximately 4S. There was, however, a contaminant nuclease activity which reduced the RNA peaks to half within one week.

iii) A satisfactory isolation procedure for ribosomal RNA and 'rapidly labelled' RNA, giving stable preparations, was found in method 2 of Kirby (206).

Ice-cooled livers were homogenized in a mixture of 15 volumes each of sodium 4-amino salicylate (6% w/v), sodium chloride (1% w/v), and a phenol cresol mixture (500 g phenol, 70 ml m-cresol, 55 ml $H_2O$, 0.5 g 8-hydroxy quinoline).

The suspension was stirred for 60 minutes at room temperature. The aqueous phase, after 30 minutes centrifugation at 600 x g, was treated with solid NaCl (3 g/100 ml aqueous phase) and 0.5 volume of the phenol-cresol mixture at room temperature for 20 minutes. The mixture was centrifuged at 8,000 x g for 15 minutes and the resulting aqueous phase mixed with twice its volume of ethanol-cresol (9:1 v/v) and allowed to stand at $4^{o}C$ for 60 minutes (or overnight). The precipitate was centrifuged off and extracted twice with cold 3 M potassium acetate pH = 6.0. This removed glycogen, DNA and most of the sRNA. The ribosomal RNA remaining insoluble under these conditions, was spun down each time at 8,000 x g for 15 minutes. The final precipitate was washed once with a cold solution of NaCl (1% v/v) in ethanol-water 3:1 (v/v), followed by one wash with ethanol-water 3:1 (v/v) and finally two with ethanol. The resulting RNA precipitate was taken up in 0.1 M NaCl - $10^{-3}$ M EDTA buffered to pH = 6.2 with sodium hydroxide (207). RNA concentrations were determined from absorption readings at 260 mμ using the extinction coefficient

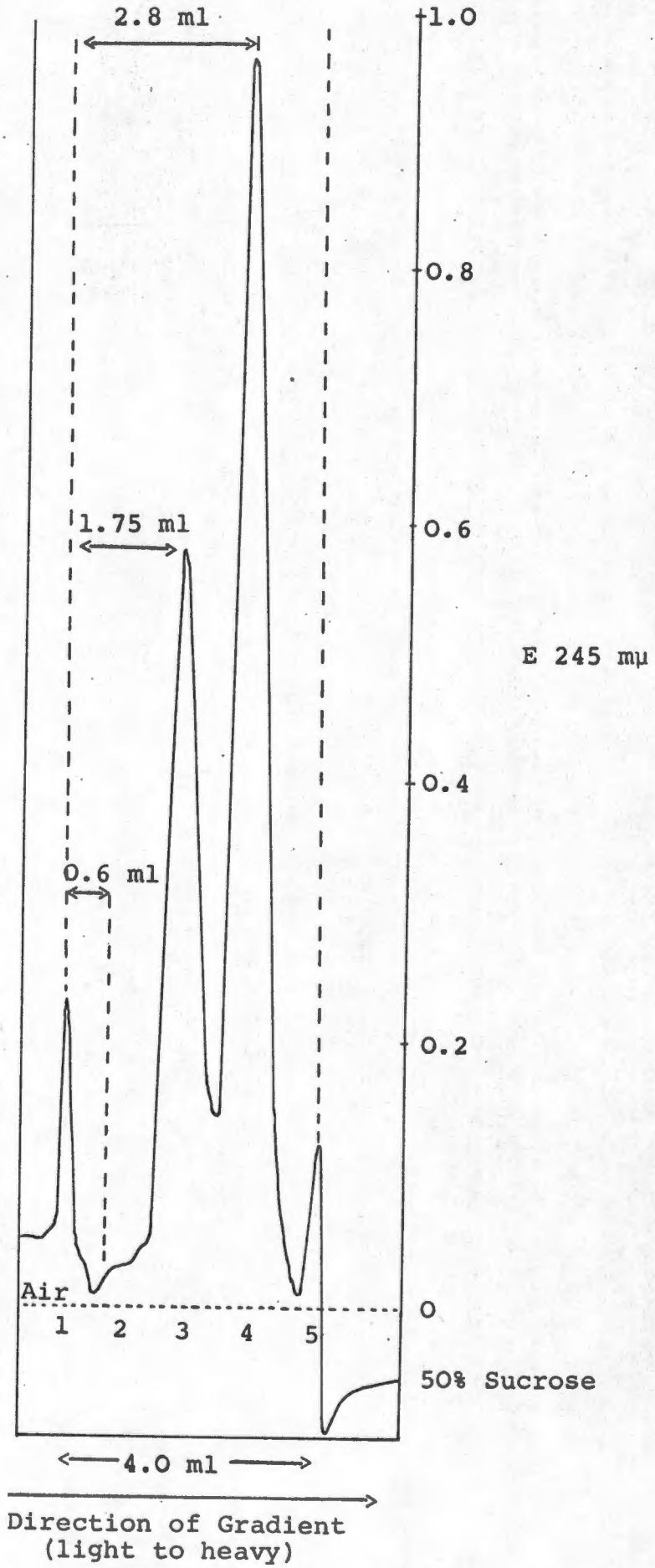
$$E \begin{matrix} 1 \text{ mg/ml} \\ 260 \text{ m}\mu \end{matrix} = 24.$$

Samples were diluted to appropriate concentration for application to sucrose gradients (3.2.2) and polyacrylamide gels (207). The molecular weight distribution of these RNA samples was identical to that of samples isolated by the method of Moldave (204), and no nuclease activity was found in these preparations.

### 3. 2. 2. SUCROSE GRADIENT CENTRIFUGATION

Linear sucrose gradients, 5 - 20% (w/v) in 0.01 M acetic acid - 0.1 M sodium chloride adjusted to pH = 5.0 with sodium hydroxide were prepared according to Krüh (208). Gradients were allowed to stabilize in the centrifuge tubes for 30 minutes at $4^{o}C$ before application of 25 μl (75 μg) RNA samples. The tubes were centrifuged at 35,000 rpm (100,000 x g) for 3½ hours at $3^{o}C$ in a SW 50 swing bucket rotor of the Spinco Model L Ultracentrifuge, removed from the rotor and positioned on an Isco Model 180 Density Gradient Fractionator. The tubes were punctured approximately 0.7 cm from the bottom with a BD22 syringe needle through which the 50% sucrose chase solution was provided to force the gradient upwards through the flow cell of the UV Analyzer.

Figure 3.3. TYPICAL PATTERN OF RIBOSOMAL RNA FRACTIONATED BY SUCROSE GRADIENT CENTRIFUGATION




Peak 1. = Artefact peak (diffraction)
= Beginning of the gradient
Peak 2. = 3-5S RNA
Peak 3. = 18S rRNA
Peak 4. = 30S rRNA
Peak 5. = Artefact peak

5-20% Sucrose gradients (w/v) were centrifuged at 100 000 x g for 3½ hours and fractionated on the density gradient fractionator (see 3.2.2). The density gradient fractionator and the recorder were run at constant speeds viz. 0.5 ml/min. and 5 mm/min. respectively. 1 cm on the recorder graph therefore equals 1 ml of the gradient.

A standard speed of 0.5 ml/minute was maintained on the fractionator and 5 mm/minute on the Metrohm Labograph E478 Recorder. The UV Analyzer was set at 254 mμ with a maximum scale of 0 - 1.0 OD.

The rising meniscus of the top of the gradient caused an artefact peak which was due to a change of diffraction, and was conveniently taken as the beginning of gradient on the recorder graph. Similarly, the meniscus of the 50% sucrose chase solution caused another such peak at the end of the gradient. Optical density readings were taken directly from the recorder graph, and when radioactive samples were being handled, fractions emerging from the flow cell were collected directly into scintillation vials. As the density gradient fractionator forces the gradient upwards at a constant rate of 0.5 ml/minute, fractions were collected on a time basis - 30 second/sample = 0.25 ml/sample. The radioactivity per fraction could then be correlated with the optical density readings from the recorder. Figure 3.3. shows a typical optical density pattern of rRNA fractionated by this method. S values were calculated from the standard ribosomal RNA 18S and 30S peaks by the equation

$$\frac{dx}{dy} = \frac{Sx}{Sy} \quad (209)$$

where dx = distance travelled by the reference RNA from the top of the gradient in ml.

dy = distance travelled by the unknown species in ml.

Sx = S value of the reference RNA

Sy = S value of the unknown species.

Specific activities of the collected samples were expressed in terms of dpm/OD on the basis of OD being a measure of RNA concentration per fraction.

## 3. 3. HISTONE PURIFICATION

### 3. 3. 1. ISOLATION OF NUCLEI (210)

A 33% rat liver homogenate was prepared in a Potter Elvehjem homogenizer with teflon pestle in 0.33 M Sucrose - 0.004 M $CaCl_2$ ($4^{\circ}C$) and centrifuged at 600 x g for 15 minutes. The sediment containing the nuclei was homogenized in three times its volume 0.25 M Sucrose-0.003 M $CaCl_2$ and layered over an equal volume of 0.34 M Sucrose-0.003 M $CaCl_2$ then spun at 1,500 x g for 15 minutes.

The supernatant was discarded and the pellet homogenized in nine times its volume 2.4 M Sucrose-0.003 M $CaCl_2$; the pelleted nuclei resulting from 70 minutes centrifugation at 45,000 x g were washed in a medium containing 0.25 M Sucrose, 0.025 M KCl, 0.01 M $MgCl_2$, 0.05 M Tris, adjusted to pH 7.6 with 1 N HCl. This yielded a white pellet of nuclei which, on microscopic examination, were found to be contaminated only slightly with erythrocytes.

### 3. 3. 2. EXTRACTION OF HISTONE

The histones isolated either directly from purified nuclei or from crude nucleoproteins prepared by citrate buffer treatment (0.14 M NaCl, 0.01 M Tri-sodium citrate pH = 7.5) of the nuclei (211) were found to be identical on polyacrylamide gel electrophoresis. Histones were therefore routinely extracted from the nuclei without prior isolation of chromatin. Nuclei were homogenized in 6 ml 0.25 N hydrochloric acid (4°C) and extracted for 1 hour at 4°C. Debris was spun off at 12,000 x g (10,000 rpm) for 15 minutes and the supernatant used for further purification.

The HCl-extract was brought to 14% trichloroacetic acid (TCA) with 1 ml 100% TCA (w/v) and allowed to stand for 15 minutes. The precipitate collected by 3 minutes centrifugation in a desk centrifuge was suspended with stirring in 6.0 ml 0.25 N hydrochloric acid (4°C). After 30 minutes insoluble material was spun off, and the supernatant brought to 25% TCA and again allowed to stand for 15 minutes. The precipitate collected as above was redissolved in 2.0 ml 0.25 N hydrochloric acid and, as before, insoluble material centrifuged off. The resulting supernatant contained the purified histones (see 3.3.3).

### 3. 3. 3. PURITY OF THE HISTONE SOLUTION

The purity of the histone preparation was judged on the basis of polyacrylamide gel electrophoresis according to the method of Panyim & Chalkley (212) on 15% gels containing 2.5 M urea. Pre-electrophoresis was run for 2 hours at 2mA/gel for gels in 10 cm long glass tubes using 0.9 N acetic acid solution as electrolyte.

After application of samples, which were dissolved either in 6 M urea containing 0.15 mercaptoethanol or brought to 15% sucrose, electrophoresis was continued for 2 - 3 hours at 2mA/gel. Gels were stained with 0.1% (w/v) Amido Black in 25% (v/v) ethanol, 7% (v/v) acetic acid and destained in a solution of 25% ethanol, 7% acetic acid. The patterns of samples under investigation were compared with those of calf thymus histone prepared according to the method of Busch et. al. (211); these yield the 5 characteristic bands as seen in Figure 3.4.(a).

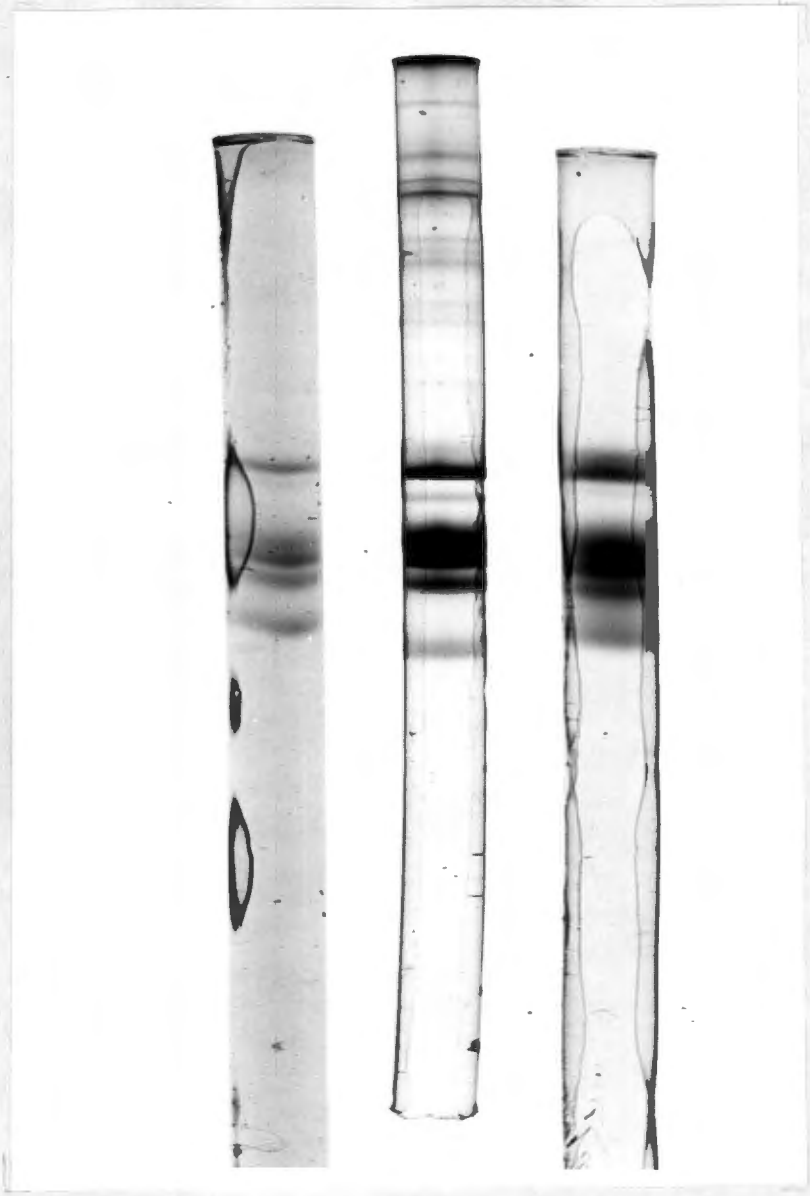
Samples for electrophoresis were taken from each purification step, and, where radioactive histones were tested, protein content (216) and radioactivities were also determined, (Figure 3.5 Schematic representation).

Electrophoresis of the HCl-extract showed the 5 characteristic histone bands and in addition a large number of contaminant bands (see figure 3.4.(b) ). Most of these contaminants, which were not identified, precipitated with the histone at TCA concentrations from 0.5 - 25% (v/v), but their trichloroacetates were subsequently less soluble in 0.25 N hydrochloric acid in which the histone dissolved. This property of the contaminants was used to purify the histones.

The first 14% TCA precipitate had an electrophoretic pattern similar to that of the original HCl-extract, but the contaminant bands were fainter. The precipitate, taken up in a 0.25 N HCl volume equal to that of the original extract contained considerable amounts of material which would not dissolve in this acid. The insoluble material was centrifuged off, resuspended in 0.25 N HCl, and the soluble part subjected to electrophoresis revealing histones heavily contaminated with slow-moving fractions.

The supernatant containing the HCl soluble material of the first TCA precipitate was brought to 25% TCA, and allowed to stand for 15 minutes. The resulting precipitate was collected by centrifugation in a desk centrifuge at a maximum speed (approximately 3,000 rpm) for 2 minutes.

Figure 3. 4. POLYACRYLAMIDE GEL ELECTROPHORESIS OF HISTONE



(a) (b) (c)

a) Histone isolated by the method of Busch et. al. (211)

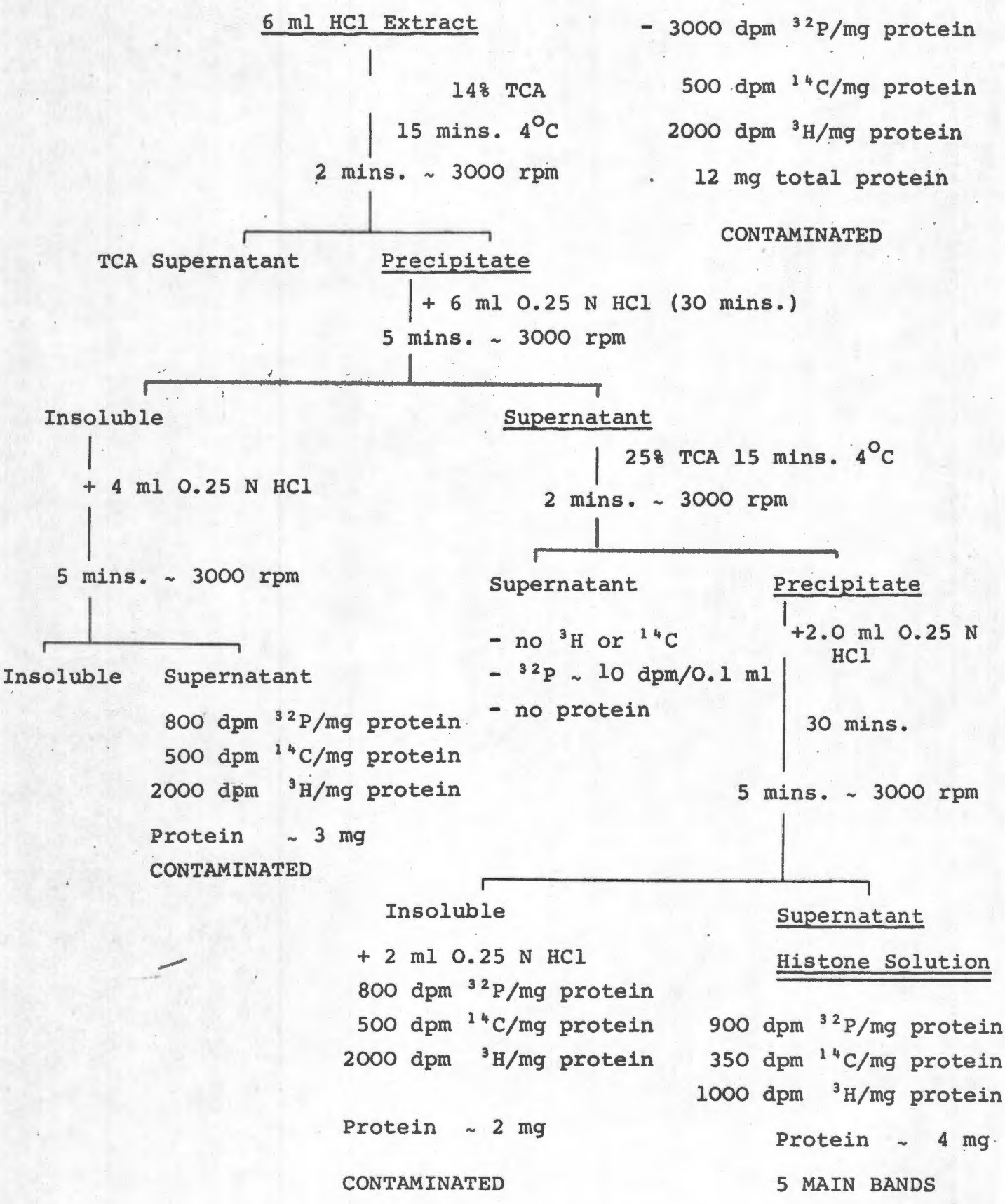
b) HCl extract showing contaminant bands.

c) Histone isolated as described in methods (3.3.2).

Electrophoresis : 2 hours at 2mA/gel, 0.9 N acetic acid electrolyte.

Staining : 30 minutes in 0.1% (w/v) Amido Black in 25% ethanol, 7% acetic acid.

Figure 3.5. PURIFICATION OF CRUDE HISTONE EXTRACT




Distribution of radioactivity during purification of histones isolated from a control rat after triple labelling (i.e. 50 µCi $^{3}$H-methionine, 80 µCi $^{14}$C-acetate, and 400 µCi $^{32}$P/200-250 g body weight).

The underlined steps represent the final purification procedure.

The TCA precipitate was taken up in 0.25 N HCl (one third of the original HCl extract volume) and insoluble material was spun off. This HCl supernatant on electrophoresis showed qualitatively an identical composition to that of calf thymus histone, and even at very high sample concentrations showed no protein contaminant (Figure 3.4.(c) ).

The HCl insoluble material of the last TCA precipitate, however, resuspended in 0.25 N HCl and subjected to electrophoresis contained, in addition to histone, the contaminants seen in the original HCl extract.

With electrophoresis as a criterion it could be seen that the repeated TCA precipitation effectively removed protein contaminants. The decrease in specific activities of the final histone solutions as compared with those of the crude histone extracts indicates that some of the contaminants were labelled (Figure 3.5). The reprecipitation, however, also led to losses of histone, possibly altering the five main fractions to differing degrees since the various histones are known to have different solubilities in TCA (297).

### 3. 3. 4. RADIOCHEMICAL PURITY OF METHYLATED HISTONE

The methylation of tRNA (267) has been shown to increase at times of increased genetic activity (268,269). Low molecular weight RNA has been detected in close association with chromatin - Bonner et. al. (142,143) have imparted a specific repressive rôle on this 'chromosomal RNA'; other workers (144-146) regard it as contaminating tRNA.

To ascertain whether the content of radioactive methyl groups in the histone preparation was due to a contamination by RNA or represented a genuine histone methylation, our methylated histone samples were tested for the possible presence of contaminating RNA.

a) RNase treatment :

Samples were subjected to RNase and the rate of reaction followed spectrophotometrically (285). Whereas control experiments with added RNA as substrate gave the expected results, no change of OD was observed when histone served as substrate. This indicates that, within the sensitivity range of the Kunitz method, the histone preparations were free of digestible RNA.

Table 3. 1.

RECOVERY OF PROTEIN AND RADIOACTIVITY AFTER RNase AND TRYPSIN TREATMENT OF METHYLATED HISTONE

| Sample | % Recovery (of total added) | | | | |
|---|---|---|---|---|---|
| | TCA Supernatant | | Precipitate | | |
| | Protein | $^3$H dpm | Protein | $^3$H dpm | |
| Zero time | 25 | 42 | 75 | 58 | RNase |
| Incubated 1. | 30 | 43 | 70 | 67 | |
| 2. | 27 | 34 | 73 | 66 | |
| Zero time | 25 | 43 | 75 | 57 | Trypsin |
| Incubated 1. | 75 | 69 | 25 | 31 | |
| 2. | 65 | 74 | 35 | 26 | |

Further samples were passed over a Sephadex G50 column (using 0.01 N HCl - 0.9% NaCl (w/v) as eluent) before and after RNase treatment. Should contaminating RNA have been present, labelled low molecular weight nucleotides would be separated from the higher molecular weight histone. 18 - 36 Hours incubation of the histone at room temperature (284) however, resulted in neither a shift of the protein peak towards the inner volume, nor the appearance of any radioactivity within the inner volume (Figure 3.6).

1 mg methylated histone in 0.25 N HCl buffered to pH = 5 with 1 N NaOH was incubated with 6.65 µg RNase (Sigma Grade 1 bovine pancreas RNase EC 2.7.7.16) in 0.1 M potassium acetate buffered to pH = 5 with 100% acetic acid (284) for 20 minutes at 37°C. The reaction was terminated by addition of TCA (6.5% final concentration) and the mixture allowed to stand at 4°C for 15 minutes. The precipitate was centrifuged off at 8,000 x g for 15 minutes. The TCA supernatant was decanted off and the precipitate taken up in 1 ml pH = 7.6 buffer (see Trypsin digestion). The radioactivity and protein content (216) of each were then determined.

As histones are partially soluble in TCA (see above 3.3.3) the TCA precipitation of protein was incomplete (Table 3.1). Nevertheless, it is evident that RNase treatment of histone did not alter the percentage recovery of Lowry positive material (216) in the TCA supernatant nor lead to an increase of radioactivity in this supernatant (Table 3.1).

b) Trypsin digestion :

1 mg methylated histone in 0.25 N HCl buffered to pH = 7.6 with 1 N NaOH was incubated with 5 µg trypsin (Seravac Grade 1 bovine pancreas trypsin EC 3.4.4.4.) in 0.2 M borate - 0.005 M $CaCl_2$ adjusted to pH = 7.6 with 1 N HCl (286) for 20 minutes at 37°C. TCA precipitation was carried out as described above (RNase treatment). The pronounced increase of radioactivity and Lowry positive material in the TCA supernatant indicated the presence of labelled peptide (Table 3.1).

Figure 3.6. COLUMN CHROMATOGRAPHY OF RNA AND HISTONE BEFORE AND AFTER RNase TREATMENT

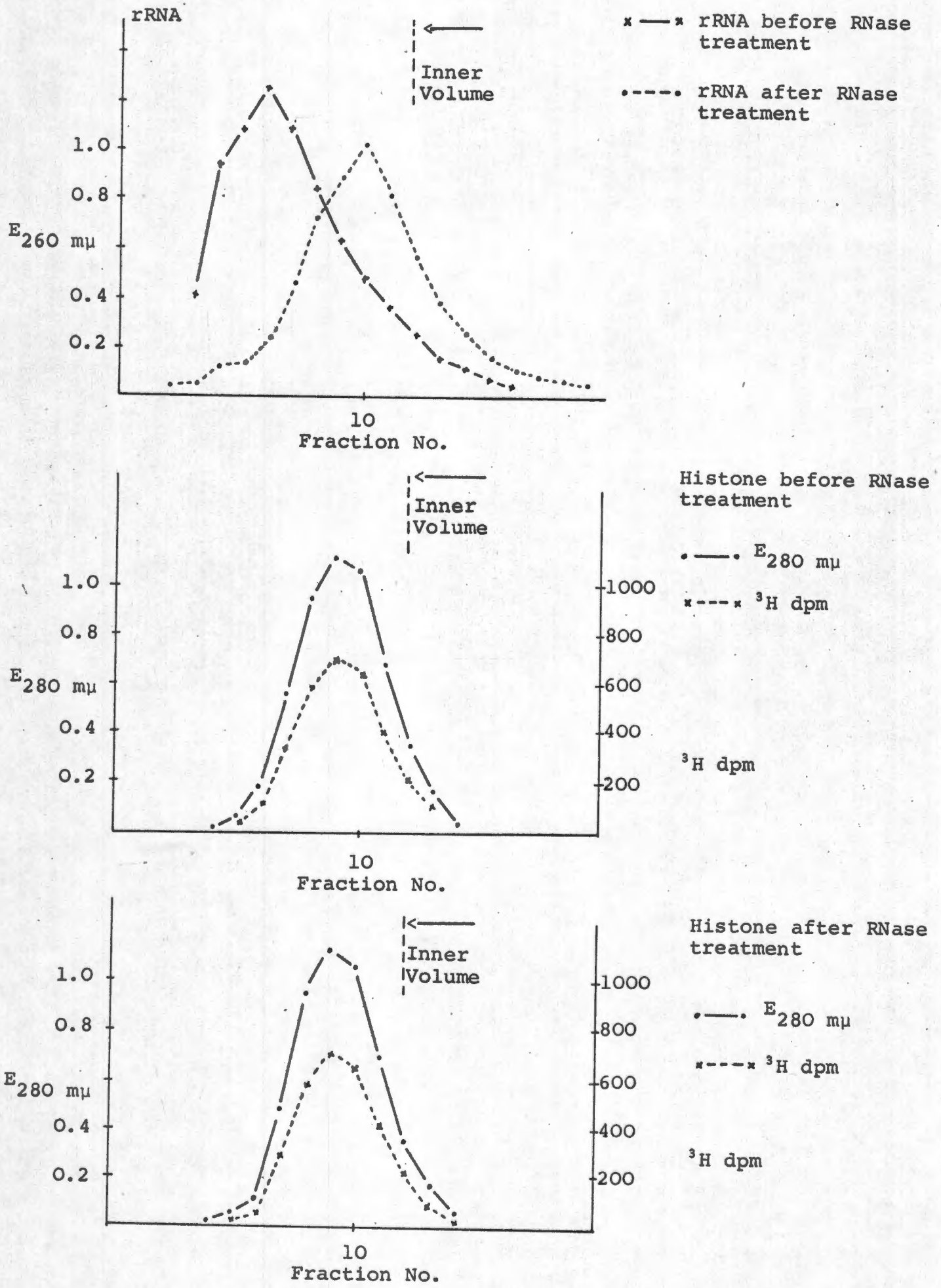
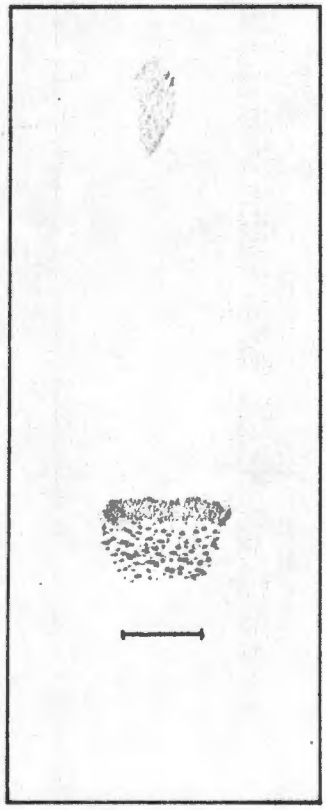


Figure 3.7. HIGH VOLTAGE ELECTROPHORESIS OF TRYPSIN DIGESTS OF METHYLATED HISTONE



61.0 $^{3}$H dpm — Purple

50.0 $^{3}$H dpm — Purple

83.0 $^{3}$H dpm — Orange

Ninhydrin stain

29.0 $^{3}$H dpm — Origin

total 223.0 $^{3}$H dpm

Sample : TCA supernatant of trypsin digest (see p. 28) desalted by passing it over a Dowex 50 (X4) $H^{+}$ column, and drying of the $NH_4OH$ eluent under nitrogen.

Total radioactivity = 277.0 $^{3}$H dpm

High Voltage Electrophoresis : 60 minutes, 3000 V

Pyridine-acetate buffer pH = 3.6 (299)

Trypsin digests were subjected to high voltage paper electrophoresis using pyridine-acetate buffer pH = 3.6 (299) as mobile phase. The TCA supernatant of the digest described above was desalted by passing it over a Dowex 50 (X4) $H^+$ column; the water eluent, devoid of radioactivity, was discarded and the 1 N $NH_4OH$ eluent dried under nitrogen. The resulting material (277.0 $^3H$ dpm) was applied and electrophoresis continued for 60 minutes at 3000 V. Dried electrophoretograms were stained with ninhydrin (300 mg ninhydrin, 3 ml glacial acetic acid, 100 ml ethanol) and stained portions cut out for determination of radioactivity. Under the conditions chosen histones remained at the origin, and RNA or oligonucleotides because of their charge at this pH would also remain stationary or move toward the cathode. The ninhydrin positive regions returned 80% of the radioactivity applied. (Figure 3.7).

c) Acid hydrolysis :

Labelled histone samples were hydrolyzed in 6 N distilled HCl for 24 hours at 110°C, and the hydrolysates applied to a Dowex 50 (X4) $H^+$ column. Any contaminating RNA or nucleotide would appear in the water eluent - this contained no radioactivity. The 1 N $NH_4OH$ eluent in which degraded peptides and amino acids would be found, contained 100% of the radioactivity applied to the column.

The methylated histone samples were therefore not contaminated with RNA and the content of radioactive methyl groups indeed represented a true histone methylation.

## 3. 4. SCHEDULE OF TREATMENT OF EXPERIMENTAL ANIMALS

Hepatic tyrosine transaminase activity is known to vary during each 24 hour period in untreated rats given free access to dietry protein and exposed to alternate periods of light and darkness (213,214,215). In addition, increases of protein in the diet of experimental animals give rise to increases in the activities of liver enzymes (3). Standard starvation periods of 20 hours were therefore observed : In all cases the male albino rats (200-250 g body weight) were fasted from 1.00 p.m. of the preceding day until 9.00 a.m. of the day of the experiment. Hormone (either hydrocortisone 10 mg/200-250 g rat) or insulin (0.05 mg in 0.1 N hydrochloric acid/200-250 g rat) was administered by intraperitonaeal injection, and induction periods were taken from this time. Starvation continued until the time of killing. The animals were stunned by a blow on the head, decapitated, and exsanguinated before removal of the livers.

TAT enzyme extracts were prepared and assayed immediately (see 3.1.2.ii).

RNA synthesis was investigated by measuring the incorporation of radioactive precursor : 250 µCi Adenine-2-$^{3}$H was administered by intraperitonaeal injection to each 200-250 g animal exactly 1 hour before sacrifice.

Methylation, acetylation and phosphorylation of total histone were measured by the incorporation of ($^{3}$H-methyl) methionine, sodium acetate-1-$^{14}$C, and $^{32}$P respectively. Induction periods from 15 minutes to 6 hours were investigated and in all cases radioactivity was injected intraperitonaeally 15 minutes before sacrifice.

Standard doses were :

i) Methionine-($^{3}$H-methyl), aqueous solution 1 mCi/ml
100 mCi/mmole
50 µCi/200-250 g body weight

ii) Na-acetate-1-$^{14}$C, aqueous solution 1 mCi/5 ml
59 mCi/mmole
80 µCi/200-250 g body weight

(Methionine and acetate were combined for injection in a cocktail containing 2.0 ml $^{14}$C-acetate + 0.25 ml $^{3}$H-methionine for 4 animals. 0.45 ml was injected
= 50 µCi $^{3}$H-methionine + 80 µCi $^{14}$C-acetate).

iii) $^{32}$P Phosphoric acid
400 µCi/200-250 g animal.

In cases of triple isotope labelling, $^{32}$P was injected separately, in volumes varying according to the age of the $^{32}$P solution.

Control animals, injected only with radioactivity, were investigated with the hormone-treated animals at each time.

## 3. 5. RADIOACTIVITY DETERMINATIONS

All samples were counted in 10 ml of toluene scintillation cocktail comprising 0.8% TLA (Beckman fluoralloy formula TLA) and concentrations of BBS-3 (Beckman Biosolv solubilizer formula BBS-3) depending on the type and size of the sample to be counted.

0.4 ml 1 N $NH_4OH$ eluent (TAT assay) : 10% BBS-3 (see 3.1.2).

0.25 ml sucrose solution (gradient centrifugation) : 6% BBS-3
3% $H_2O$ (see 3.2.2).

0.1 ml 0.25 N HCl solution of histones : 2.5% BBS-3 (see 3.3.2).

The radioactivity of the samples was determined using appropriate discriminator settings with a Beckman Model LS 250 Liquid Scintillation Counter to 3% error. Cpm, corrected for background, were converted to dpm by use of quench curves built up for this system and a computer programme correcting for $^{32}P$ "spillover" into $^{14}C$ and $^{3}H$ channels, for $^{14}C$ "spillover" into the $^{3}H$ channel, and for decay of $^{32}P$ (298).

To compensate for variations in the specific activity of the precursor pools in the different animals as a result of weight variations (from 200-250 g) yet a constant dose of radioactivity, all radioactivity data were corrected to an average liver weight of 6 g. A direct proportionality between liver weight and body weight does probably not occur. However, any attempt to achieve equal labelling of the precursor pool by adjusting the dosage of the already small volume (0.45 ml) of highly radioactive solutions, would have introduced even greater errors in dosing.

## 4. RESULTS

In all sets of experiments the various parameters during an induction period from 0 to 6 hours were investigated. Enzyme activity, RNA synthesis and histone modification were studied separately in different groups of animals. As animals from the same stock were used, and were subjected to identical treatment prior to and during the induction periods, it was presumed that the events described at each specific time could validly be compared. Only in a few pilot runs were all three parameters measured in a single animal. The results of these experiments corresponded with those done separately in different animals.

### 4. 1. TYROSINE TRANSAMINASE :

Control animals, not receiving hormone treatment, were investigated at each time interval to account for any indogenous changes of hormonal activity which may have occurred in the course of the experiment as a result of prolonged starvation. The levels of TAT activity in these rats varied little within the experimental period apart from a very slight drop at 6 hours. This might be related to the normal diurnal variation in TAT activity (214) (see Figure 4.1a). Hydrocortisone administration had no measurable effect on the enzyme until 4 hours after injection had elapsed, whereupon the activity rose to twice that of controls. (Figure 4.1b. and Table 4.1.). A further increase to 4 times the control level was noted after 6 hours.

Hydrocortisone induction of TAT activity is well documented (4,182,184). The effect of insulin on TAT has not been comprehensively reported. Kenney et. al. (181,217) have compared hydrocortisone, glucagon and insulin induction both *in vitro* and *in vivo* . They noted that insulin injection rapidly increased the enzyme activity, which then declined to control levels despite the continuous presence of hormone. However, with hydrocortisone the high level was maintained throughout the experimental period. The authors inferred different mechanisms by which the two hormones operate.

TABLE 4.1. HYDROCORTISONE AND INSULIN INDUCTION OF TAT ACTIVITY

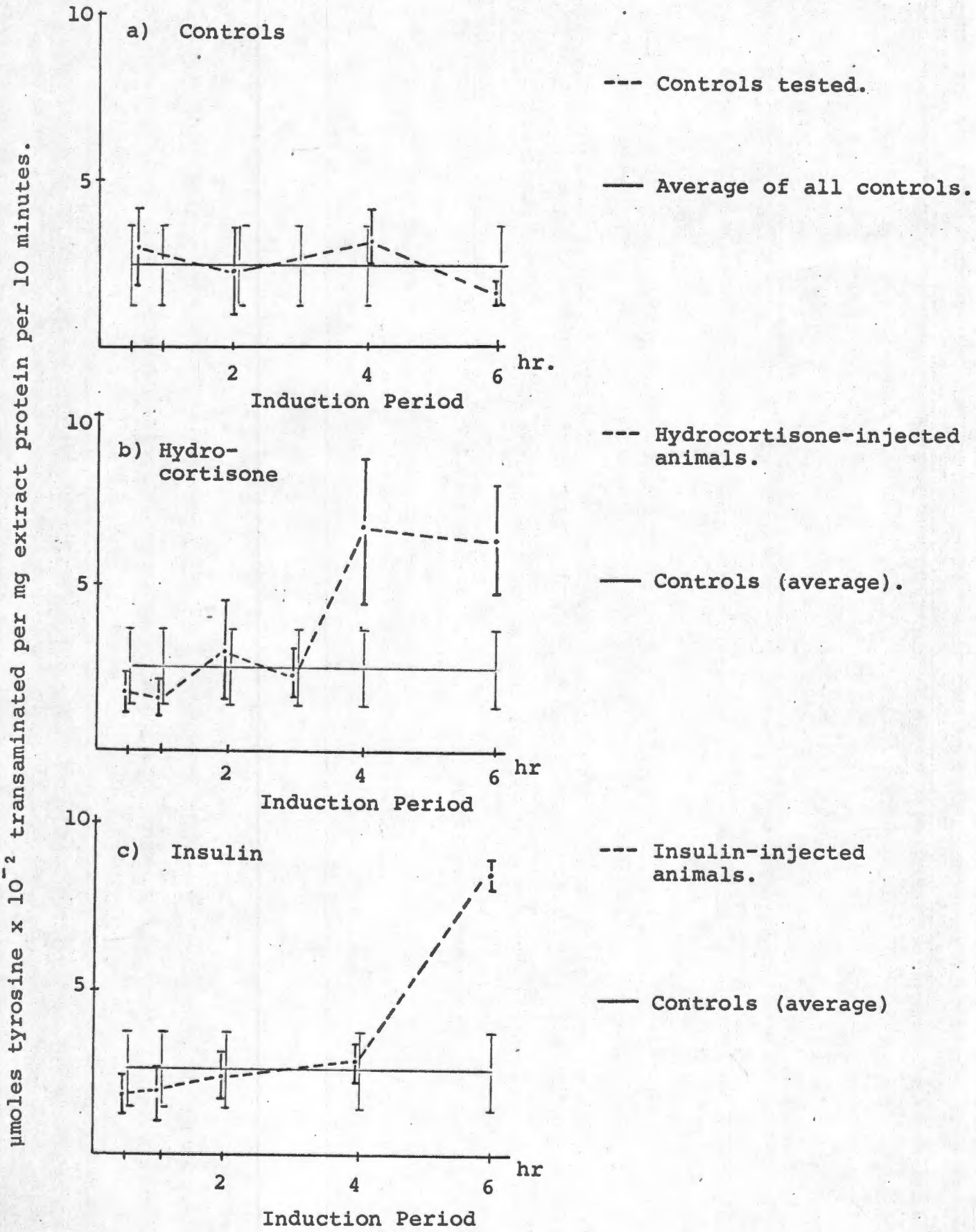
| Induction Period | TAT Activity (μmoles transaminated / mg protein per 10 minutes ± standard deviation). | | |
|---|---|---|---|
| | Control | Hydrocortisone | Insulin |
| 30 minutes | 0.02993 ± 0.0126 | 0.01852 ± 0.00573 | 0.01852 ± 0.00567 |
| 60 minutes | | 0.01662 ± 0.00520 | 0.01910 ± 0.00743 |
| 2 hours | 0.02431 ± 0.0134 | 0.03504 ± 0.01458 | 0.02412 ± 0.00756 |
| 3 hours | | 0.03379 ± 0.007198 | |
| 4 hours | 0.03254 ± 0.00898 | 0.06635 ± 0.02254 | 0.02773 ± 0.00664 |
| 6 hours | 0.01592 ± 0.00340* | 0.06190 ± 0.01626 | 0.08410 ± 0.004339 |
| | Average of all controls : 0.025095 ± 0.01113 | | |

Each value is the average ( ± standard deviation) of a minimum of 4 animals.

* Significance of difference between the 6 hour value and the average of all controls P = 0.1.

| Induction Period | Induction Factor: TAT activity of experimental rats / TAT activity of control rats | | Significance (P) of the difference between injected and control rats (i.e. average of all controls). | |
|---|---|---|---|---|
| | Hydrocortisone | Insulin | Hydrocortisone | Insulin |
| 30 minutes | 0.618 | 0.618 | 0.3 | 0.3 |
| 60 minutes | 0.6 | 0.8 | 0.1 | 0.2 |
| 2 hours | 1.2 | 1.0 | 0.1 | 0.9 |
| 3 hours | 1.2 | | 0.2 | - |
| 4 hours | 2.04 | 0.92 | 0.001 | 0.7 |
| 6 hours | 3.89 | 5.28 | 0.001 | 0.001 |

Figure 4.1. HORMONE INDUCTION OF TYROSINE TRANSAMINASE ACTIVITY




Each point represents the average ± standard deviation of a minimum of 4 animals.

Our experiments showed that the induction effect of insulin on TAT occurs somewhat later than that of hydrocortisone under similar conditions. Whereas the latter had already caused a two-fold increase after 4 hours, insulin induction was not manifest until after 6 hours (see Figure 4.1c, and Table 4.1). At this stage there was a five-fold increase in TAT activity.

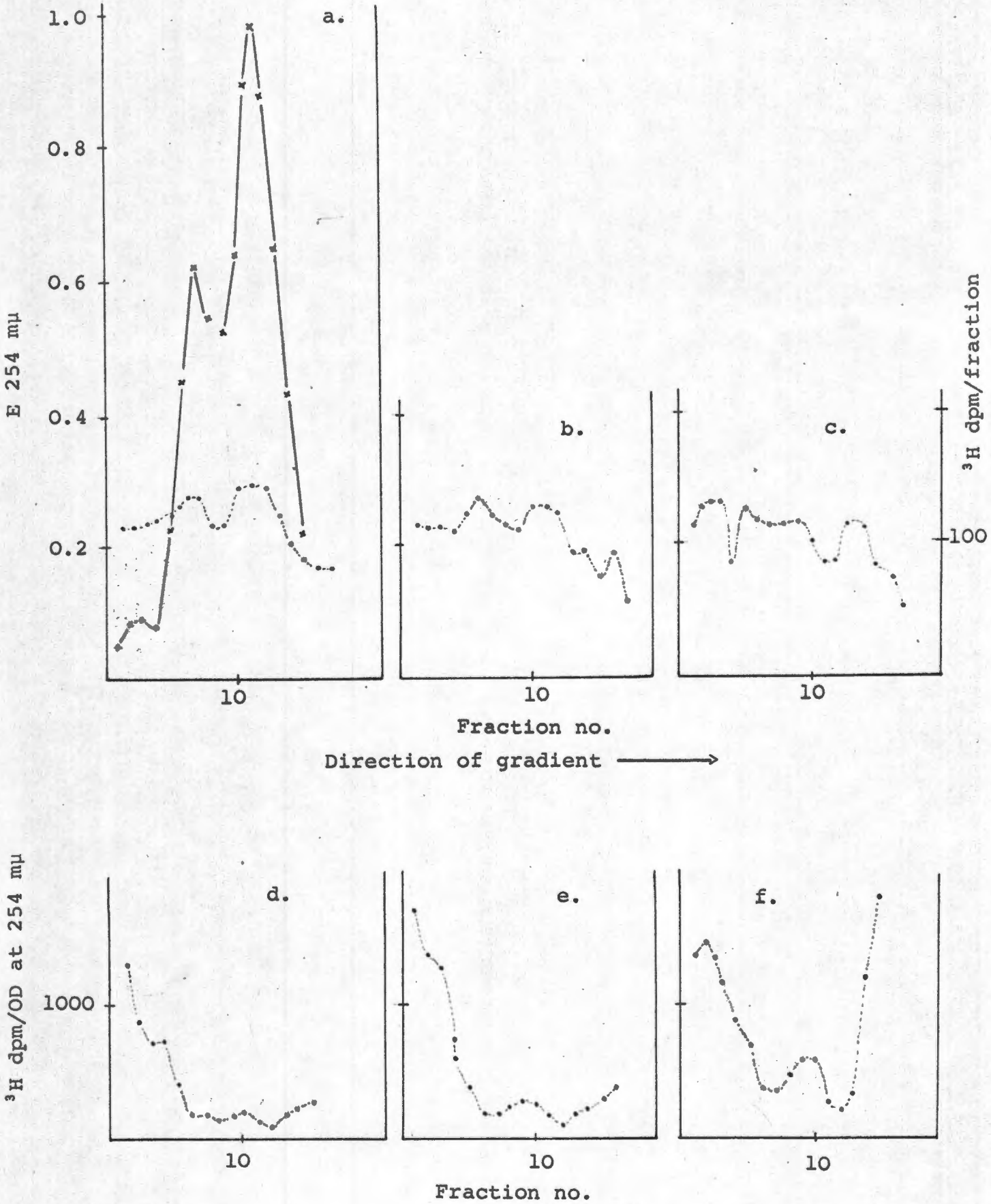
## 4.2. RNA SYNTHESIS

One of the early responses in target cells to hormone treatment is the de novo synthesis of RNA in the nucleus (32). This involves the pre-ribosomal and ribosomal RNA types (162,163) and the DNA-like RNA species (173,218). The well characterised 45S ribosomal RNA precursors are transformed by a complex series of reactions to the mature 18S and 30S RNA's (164,174,175). Weinburg et. al. (175) have proposed a mechanism by which 45S pre-rRNA is converted into a 41S molecule, which is further split into immediate precursor molecules, a 32S RNA for the 28S and a 20S RNA for the 18S. Approximately 50% of the 45S RNA molecule is thus discarded during the maturation process (164,174). The high molecular weight DNA-like RNA is composed of 2 types, a non-informational species which is restricted to the nucleus (219), and an informational species which is also found in the cytoplasm (174,219). Although the mechanism of cleavage is not yet certain, a pathway has been proposed (174) following the sequence 50S——>30S——>18S——>10S. These DNA-like species have the characteristics associated with mRNA, (173,218,220), and their synthesis is stimulated by hormone to the same extent as that of rRNA (162,163).

Low molecular weight RNA's are also increased in response to hormone treatment (173). These include transfer RNA (162,173) and DNA-like molecules (221) which Kenney et. al. (173) have labelled 'high molecular weight DNA-like degradation products'. This designation would agree with the cleavage pattern of Sekeris et. al. (174) (50S——>30S——>18S——>10S).

Figure 4.2.1. $^{3}$H-ADENINE INCORPORATION INTO RNA OF CONTROL ANIMALS

Sucrose Gradient Fractionation.



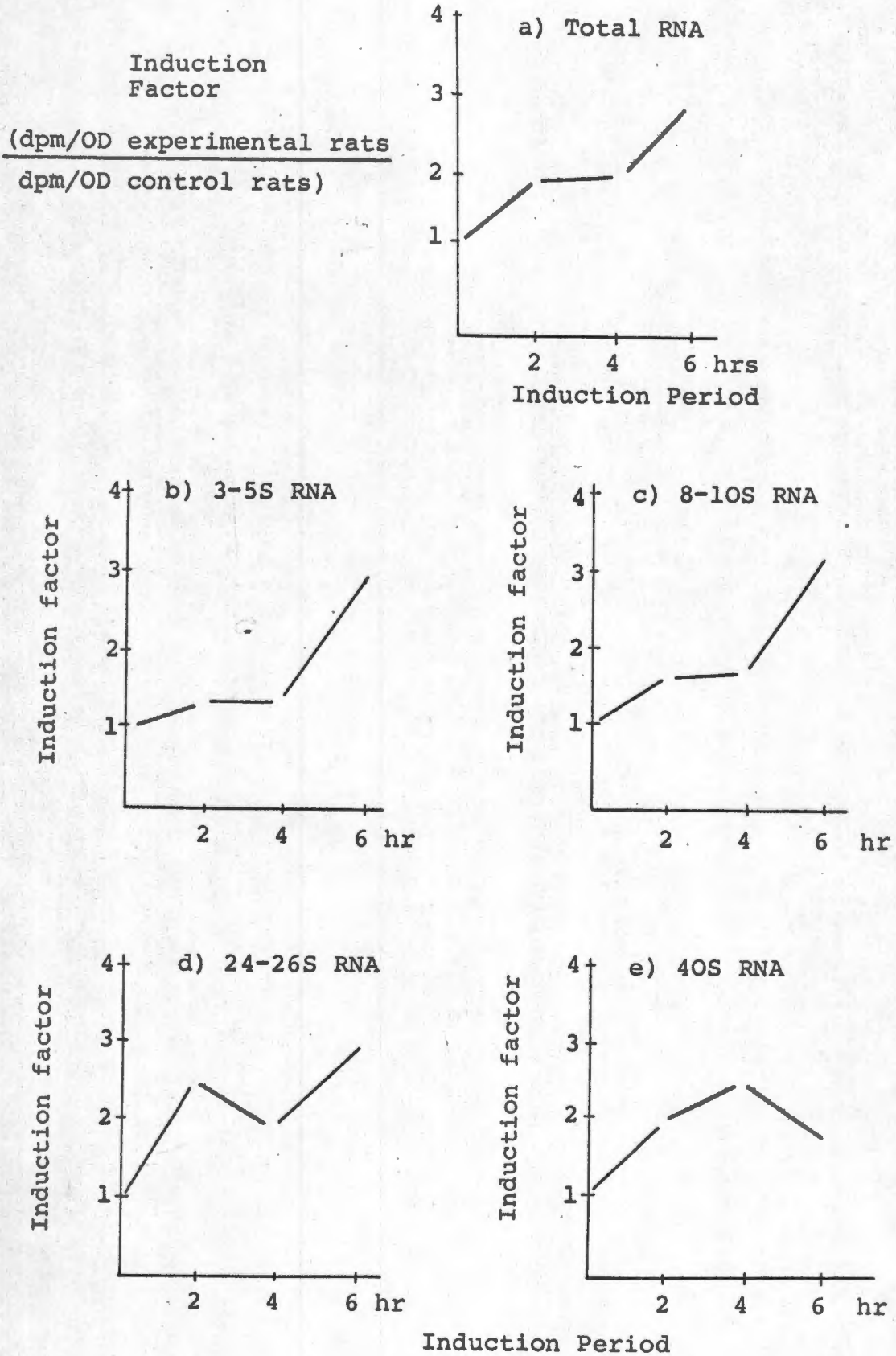

a-c ..... $^{3}$H dpm per fraction.
----- Typical OD pattern.

a,b,c, : Incorporation of radioactivity into RNA of control animals at 2, 4 and 6 hours respectively. (Each taken from an average of 4 animals).

d,e,f ... Specific activities ($^{3}$H dpm/OD) at 2, 4 and 6 hours.

Adenine - $^{3}$H (250 μCi/200-250 g rat) was injected 1 hour before

Figure 4.2.2. EFFECT OF HYDROCORTISONE ON $^{3}$H-ADENINE INCORPORATION INTO RNA FRACTIONS




Each point represents the quotient of the average of the RNA specific activities of a minimum of 6 hormone-treated animals and 4 control animals.

In the present experiments, hydrocortisone was administered, $^{3}$H-Adenine was injected, rRNA and 'rapidly labelled RNA' were isolated and fractionated as described in Methods (3.2). RNA synthesis was measured by incorporation of the labelled precursor in experimental animals 2, 4 and 6 hours after hormone injection, and in uninjected animals at 2, 4 and 6 hours.

In control rats radioactivity appeared in low molecular weight fractions (3 - 5S), the two rRNA regions (18S and 30S) and also in a high molecular weight fraction (40S) (Figure 4.2.1a-c).

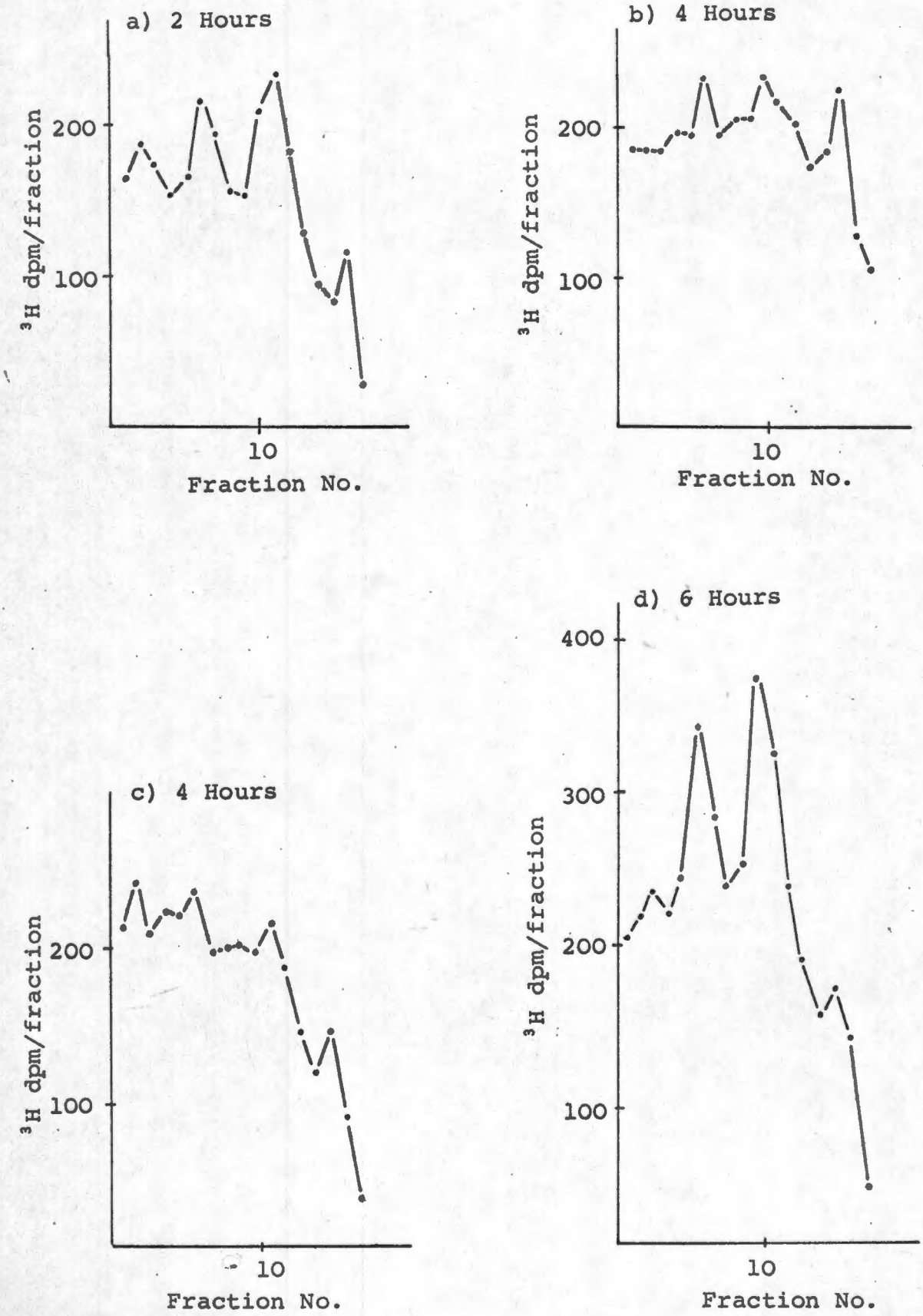
Specific activity determinations (dpm/OD. See Figure 4.2.1d-f) of the RNA isolated from control animals show that there was no significant change in incorporation into low molecular weight species throughout the experimental period. With the 16 - 30S and 40S areas regular incorporation was maintained from 2 to 4 hours, whereas at 6 hours there was a slight increase which may reflect the changeover in the rat's metabolism to starvation conditions (140).

The hormonal effect on RNA synthesis in animals injected with hydrocortisone was evident two hours after administration, when the specific activity of total RNA was twice that of controls; this level was maintained at 4 hours, after which there was a rise to 3 times control values at 6 hours (Figure 4.2.2a). No gross changes in the proportion of the amounts of the various RNA fractions were detected as the optical density profiles were not affected. The incorporation of radioactivity, however, was found to increase in all fractions (Figure 4.2.3a-d). Two slightly different patterns were observed at 4 hours : one in which the distribution of radioactivity was more or less similar to that at the 2 and 6 hour periods (Figure 4.2.3b); the other where there was an increase in the 8 - 10S region and more diffuse incorporation between 16 and 32S (Figure 4.2.3c).

By specific activity determinations, low molecular weight RNA (3 - 5S) was shown to increase only slightly above control levels at 2 and 4 hours, followed by a high incorporation at 6 hours, viz. 3 times the 6 hour control values (Figure 4.2.4).

Figure 4. 2. 3. $^{3}$H-ADENINE INCORPORATION INTO RNA OF HYDROCORTISONE INJECTED ANIMALS

Sucrose gradient fractionation : Total Incorporation.

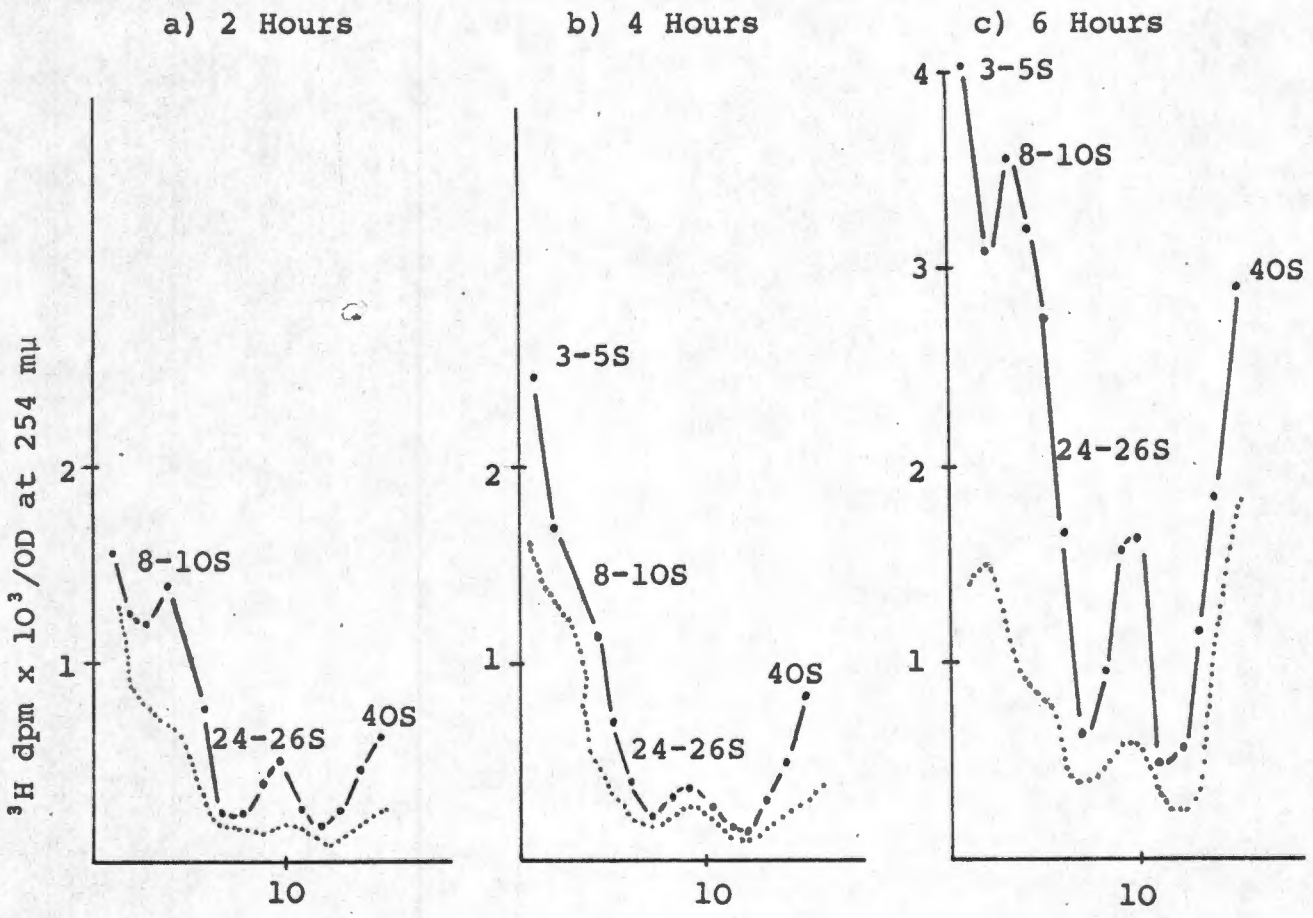



a) and d) : Average of 6 experiments

b) and c) : Single experiments each typical of the patterns obtained from at least 2 animals.

Figure 4.2.4. $^{3}$H-ADENINE INCORPORATION INTO RNA OF HYDROCORTISONE INJECTED ANIMALS

Sucrose gradient fractionation : Specific Activities



The 8 - 10S RNA was already raised at 2 hours to double the 2 hour controls. At 4 hours these species were increased two- to three times the corresponding controls (Figure 4.2.4) while at 6 hours the increase had become four-fold.

In the 18 - 30S region, where a maximum peak was seen between 24 and 26S, a similar induction pattern was found (viz. twice control values at 2 and 4 hours, three times control values at 6 hours).

In the heavy RNA region, ~40S, the two-fold rise at 2 hours increased slightly at 4 hours and fell again at 6 hours to the two hour level.

The RNA fractions isolated under the present experimental conditions were not characterised with regard to base composition. It was therefore not possible to differentiate with certainty between DNA-like RNA and other species.

Hydrocortisone effected a pronounced increase in synthesis of all types of RNA. In the 40S region containing both heavy DNA-like RNA and 45S precursor ribosomal RNA, the maximum incorporation was found 4 hours after hormone administration (Figure 4.2.2e).

Ribosomal RNA, 18S and 30S (and possibly their immediate 20S and 32S precursors (175) would comprise the 18 - 30S region which was increased to a maximum at 6 hours (Figure 4.2.2d). This was also the case for the 8 - 10S region which would contain the low molecular weight DNA-like molecules (164,173,221) (Figure 4.2.2c).

In the light RNA region (3 - 5S) where sRNA would be the major component, the early response to hydrocortisone was not as great as that of the larger RNA species. However, after 6 hours the increase was equal to that of the other regions (Figure 4.2.2b).

The chromosomal RNA of Bonner et. al. (142) would not be detected by the methods used in these experiments.

Template activity of rat liver chromatin and RNA polymerase activity are increased by administration of insulin (186) to an extent similar to that caused by hydrocortisone (7,14).

Since increased RNA synthesis is the result of both of these changes, our investigations included only one cross experiment with insulin. Two hours after administration of this hormone, the increased amount of $^{3}$H-Adenine incorporated was identical to that in response to hydrocortisone (Figure 4.2.4a).

## 4. 3. HISTONE MODIFICATION :

The possible role of histones as genetic repressors and their relationships with non-histone chromosomal proteins, DNA and RNA polymerases has already been discussed (see Section 1). The effects of hormone or other inducing agents on the structural modifications of histones remain uncertain (46). Conflicting results have been reported with regard to acetylation reactions in response to hydrocortisone (188,222), and phytohemaglutinin (37,223). Pogo et. al. (188) have found an increase in RNA synthesis concomitant with increased acetylation of arginine-rich histones in hepatic tissue of cortisol-treated rats, while Gallwitz & Sekeris (222) found no such accompanying modification of histone. Both negative (36) and positive (224) responses to hydrocortisone have been reported involving the phosphorylation of the lysine-rich histones.

The mechanism of induction by hormones has been related to :-

(i) stimulation of the activity of the enzymes responsible for histone modification (36,79)

(ii) direct interaction of hormone with histone (150,152,156)

(iii) interaction of hormone with the metabolism of non-histone chromosomal proteins and their inter-relationships with histone and RNA polymerase (112,132,134-139) (See Section 1).

On a structural basis, acetylation and phosphorylation of the basic proteins associated with chromatin would result in a weakening of the electrostatic bonds between histone and DNA (47,53,60) rendering the DNA more available for transcription (61,65) or replication (46,65). Acetylation and phosphorylation of arginine-rich histones have been related to RNA synthesis (transcription) (65,187,190,223) while the phosphorylation of lysine-rich histone f1 appears to accompany DNA synthesis (replication) (82,225-231).

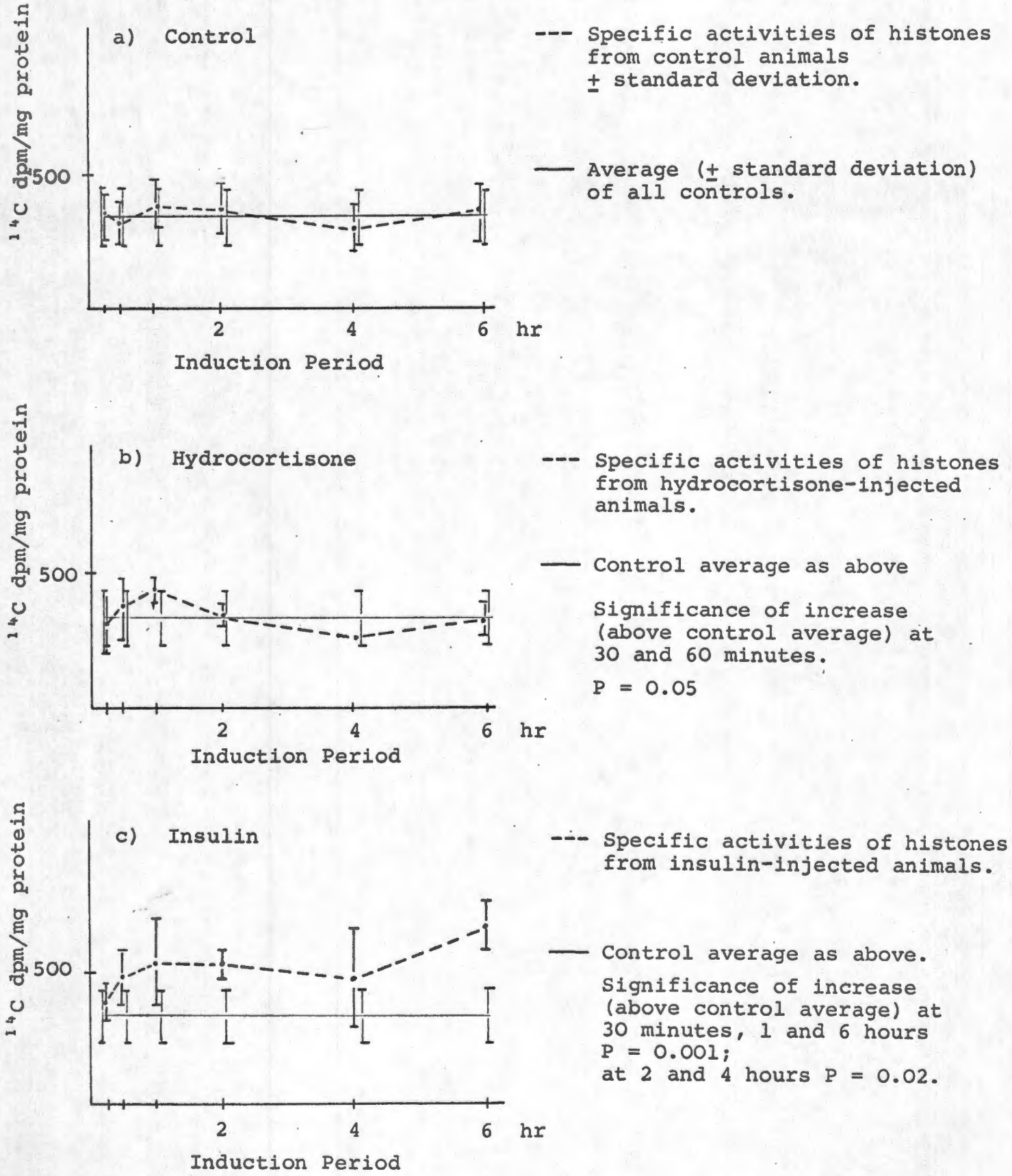
Methylation of histone would result in an increase of the positive charge, hence a tightening-up of the bonds between histone and DNA (46,63). Paik and Kim (295) have shown that, in contrast to mono- and trimethylation, dimethylation of the ε-amino groups of lysyl residues, results in a decreased basicity at the substituted group. Thus dimethylation of such residues could conceivably have a similar effect on the histone-DNA interaction to that of acetylation and phosphorylation. The function of methylation remains a mystery (66,191,232) - it may be related to a decreased availability of the genome for transcription after the latter has been completed (63,191) or increased structural stability of the nucleoprotein against damage or degradation (97,188-190,233).

4. 3. 1. ACETYLATION :

It is generally accepted that the acetylation of α-amino groups of the $NH_2$-terminal residues of histone (234) occurs in the cytoplasm (34) relatively late in the cell cycle (235) and is related to the initiation of histone biosynthesis (34,235). These acetyl groups, found in histones f1 (70,236) and f2a (67,234,237,238), do not show an appreciable turnover throughout the cell cycle (100).

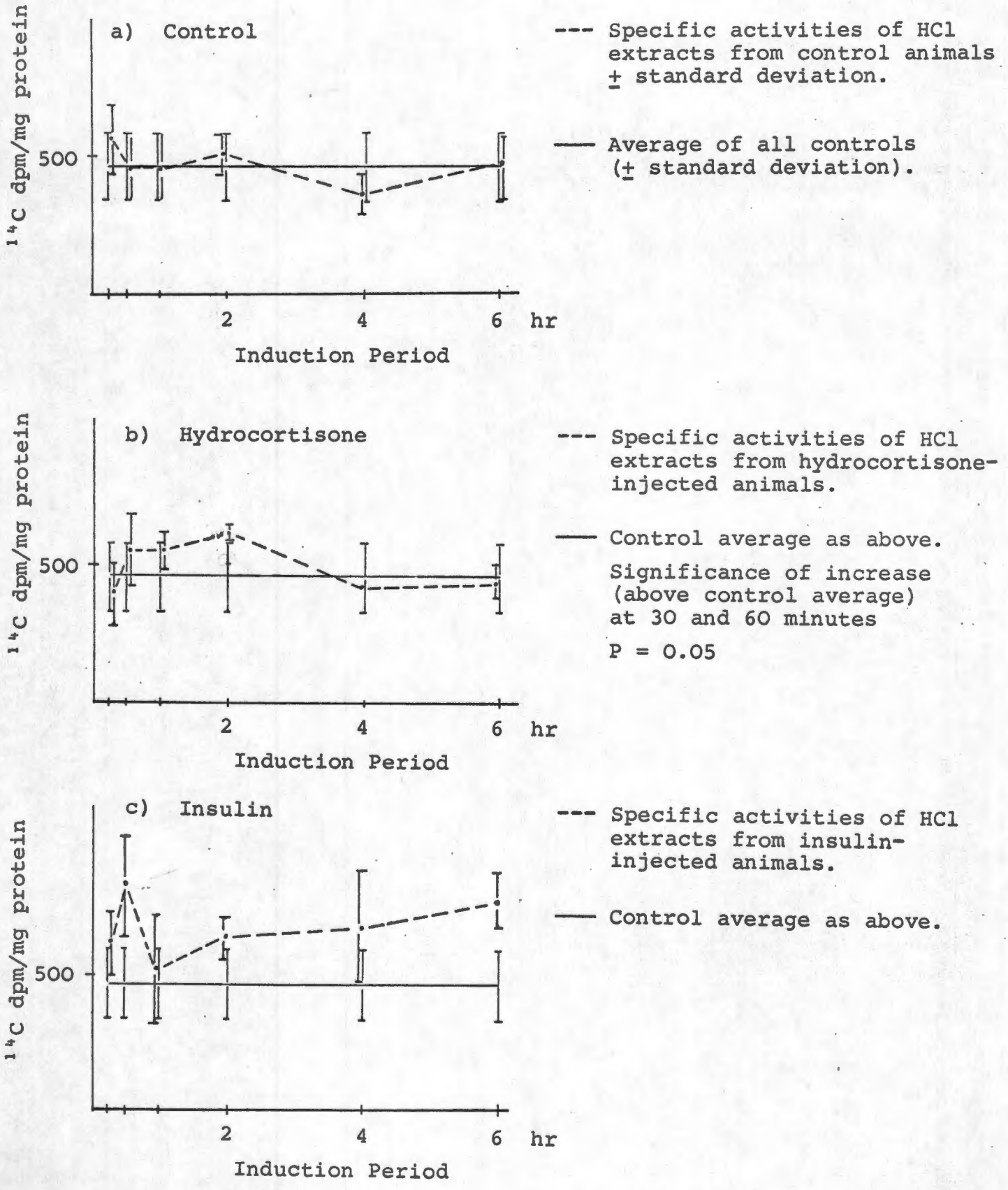
In contrast, the acetylated ε-amino groups of internal lysyl residues (67,70,97,238-240) turn over at an appreciable rate (70,101,239). This acetylation reaction occurs in the nucleus (70,239,241) after histone synthesis has been completed (72,74,97) preferentially on the arginine-rich histones (41,70,71,74,189).

Figure 4. 3. 1. EFFECT OF HYDROCORTISONE AND INSULIN ON HISTONE ACETYLATION




80 µCi $^{14}$C-acetate/200-250 g rat were injected 15 minutes before sacrifice. Each point is the average (± standard deviation) of a minimum of 3 experiments.

Figure 4. 3. 2. EFFECT OF HYDROCORTISONE AND INSULIN ON ACETYLATION OF HCl EXTRACT PROTEINS




80 µCi $^{14}$C-acetate/200-250 g rat were injected 15 minutes before sacrifice. Each point is the average (± standard deviation) of a minimum of 3 experiments.

Nuclear, histone specific (68) transacetylases (41,68,72-75) which show certain organ specificity (73) have been isolated, as have deacetylases (98). Hormone effects on these enzymes have not been reported; the transacetylases, however, are insensitive to cyclic AMP (73,79). Many studies (see above section 4.3 and 79,97) have related this nuclear acetylation reaction with RNA synthesis, the former preceding the latter (188,189,237,241,242).

In our experiments, when acetylation was investigated by the incorporation of $^{14}$C-acetic acid into total histone (see 3.4.) in the absence of intensive cell division, during the relatively early stages of induction by hormones (see 4.1., 4.2), the nuclear acetylation of the internal lysyl residues of histones has been measured.

A very slight rise in $^{14}$C incorporation seemed to occur 30 minutes after hydrocortisone administration, followed by a return to control levels during the remainder of the experimental period (Figure 4.3.1b and Table 4.2). However, under the conditions of these experiments, very small increases in acetylation induced by the hormone may well fall within the biological variation of the control animals, and the significance of the rise after 30 minutes remains questionable.

30 minutes after insulin injection, there was a 20% increase in radioactivity above control levels - this was maintained until the 6 hour period when there was a further increase to 50% above the corresponding control values.

The similarity of incorporation patterns of the HCl extracts and the histone solutions indicate that acid-soluble non-histone contaminants did not contribute to the increases in radioactivity seen under the influence of insulin (Figures 4.3.1 and 4.3.2). RNA acetylation does not occur under the conditions of histone acetylation (243) and non-histone chromosomal proteins are not readily acetylated (46).

4. 3. 2. PHOSPHORYLATION :

Since all constituents of chromatin incorporate $^{32}P$ (46,81) the results of experiments involving phosphorylation of any single component must be regarded as tentative unless stringent purification measures have been applied. Some chromosomal non-histone species remain associated with histones even through exceedingly elaborate separation procedures (81).

Non-histone chromosomal proteins (NHC proteins) are known to be present in high concentrations in the chromatin of metabolically active tissues (110,116,126-131) preferentially in regions of the chromosome most active in RNA synthesis (11). They are subject to structural modifications by means of enzymatic phosphorylation reactions (111,124,244-247) particularly at the time of gene activation (246,247), and under the influence of hormones (132,134). Moreover, their specific activities with regard to phosphate content, are higher than those of phosphorylated histones (124,140,288) and their phosphorylation is facilitated by their interaction with these basic proteins (113,114,116,288).

Although most of these acidic proteins (114,116) should have been removed from the histone preparation by HCl and TCA treatment (see 3.3.2), their presence as phosphorylated contaminants cannot be ignored. It is interesting to note, however, that Sonnenblicher et. al. (248) have found acid treatment of chromatin capable of giving histones the apparent characteristics of acidic chromosomal proteins.

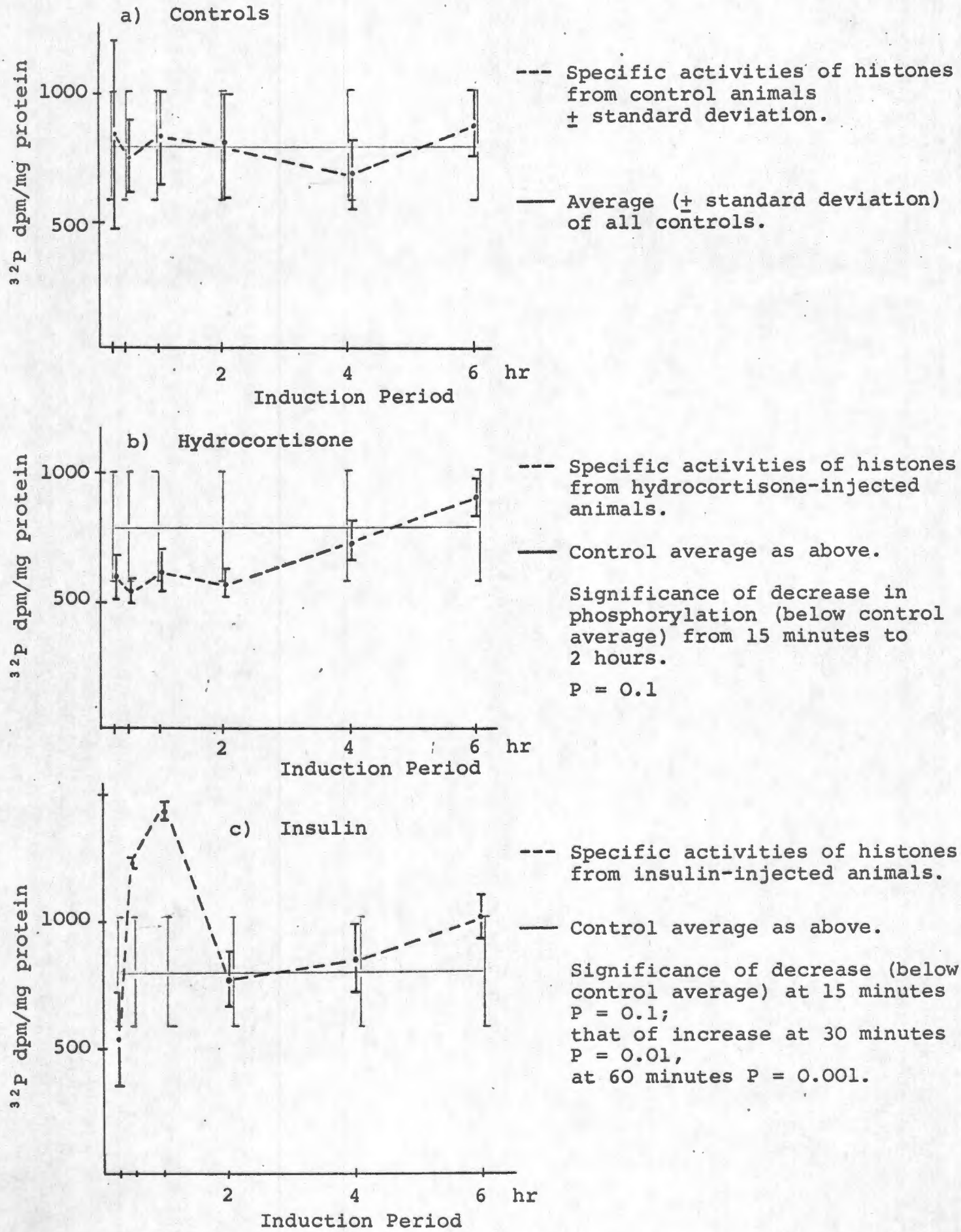
The phosphorylation of histones themselves has been extensively studied. All types are phosphorylated at least in some organisms (46,81-84) although the extent of modification of individual histone fractions varies according to biological source (249) preparation procedures (250,84) and method of phosphate incorporation (124).

Histone kinases and phosphatases must be present in tissues because of the observed uptake and turnover of histone-bound phosphate in vivo (46,61,78,86,224,247). Several protein kinases both cyclic AMP-dependent (77,79,80,251-254,276,288) and independent (80,227,276) have been found to phosphorylate histone (although not specifically (80) ), f1 being the best substrate (76-78). A histone and protamine specific phosphatase has been isolated by Meisler & Langan (99).

Both hydrocortisone (244) and insulin (36) have been reported to induce the phosphorylation of histone. The role of cyclic AMP in these reactions remains a subject under dispute (36,38,39,76,79,224,251,254,276). Correlations have been made between this modification of histones (except f1) and RNA synthesis (36-38,61,225,226,247,249,255); between the phosphorylation of f1 and RNA synthesis (65,76,190) and DNA synthesis (82,225-231). A model accounting for the effect of phosphorylation on histone - DNA interaction involving a local turning away of the protein from the DNA wide groove has been proposed by Lewin (62), the result being an increased availability of the DNA for such processes as transcription and replication.

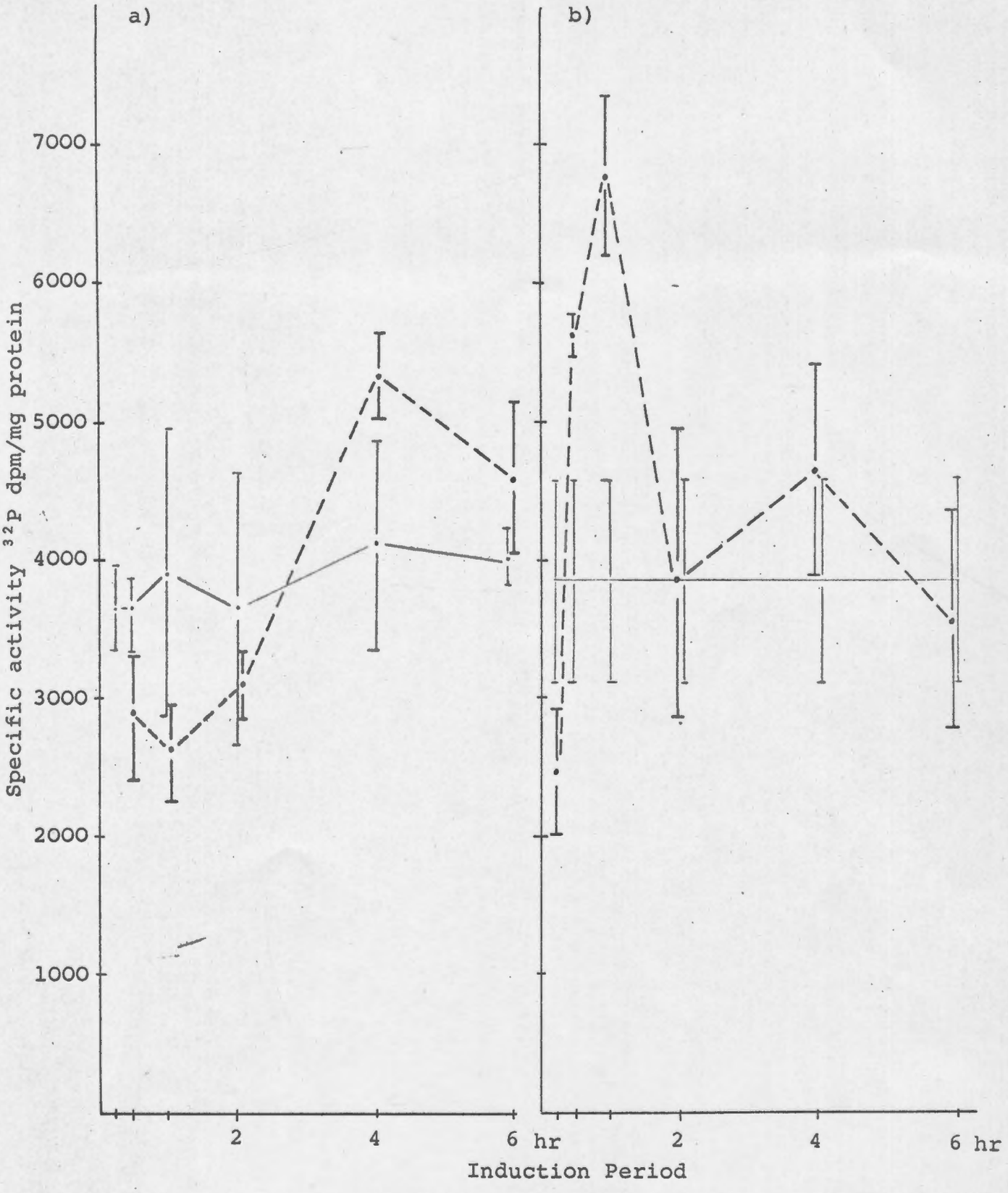
In our experiments phosphorylation of total histone was investigated by the in vivo incorporation of $^{32}P$ (see 3.4. ). The results indicate, if anything, a slight repression of incorporation after hydrocortisone administration in comparison with control animals until 2 hours after hormone injection (Figure 4.3.3b, and Table 4.2.). This effect of hydrocortisone is in agreement with the results of Langan (36) but not with those of Murthy et. al. (224), who reported an increase in histone phosphorylation in response to hydrocortisone. The incorporation into the crude HCl extract, however, indicated that non-histone components became heavily labelled after 4 hours (Figure 4.3.4a).

Figure 4. 3. 3. EFFECT OF HYDROCORTISONE AND INSULIN ON HISTONE PHOSPHORYLATION




400 µCi $^{32}$P/200-250 g rat were injected 15 minutes before sacrifice. Each point is the average (± standard deviation) of a minimum of 3 experiments.

Figure 4. 3. 4. EFFECT OF HYDROCORTISONE AND INSULIN ON PHOSPHORYLATION OF HCl EXTRACT PROTEINS




a) —— Controls ± standard deviation.

--- Hydrocortisone-injected

Significance of decrease (below control average) at
30 minutes P = 0.05
60 minutes P = 0.02

b) —— Average (± standard deviation) of all controls.

--- Insulin-injected.

Significance of decrease (below control average) at
15 minutes P = 0.02

400 µCi $^{32}P$/200-250 g rat were injected 15 minutes before sacrifice. Each point is the average (± standard deviation) of a minimum of 3 experiments.

Insulin administration caused a 50% increase in $^{32}P$ incorporation above controls within 60 minutes (see Figure 4.3.3c); the level, however, dropped to equal that of controls between 2 and 6 hours. The incorporation pattern in the HCl extract closely followed the activity in the histone fraction (Figure 4.3.4).

Contamination of the histone preparation by RNA would seem unlikely because of parallel experiments where RNA and histone were investigated from the same animal, $^{32}P$ incorporation into RNA was very low in comparison with that into histone. The specific activities of RNA would need to be extremely high to account for the observed histone radioactivity both in control and injected animals. Further, for example, at the 2 hour period where RNA incorporation of labelled adenine had increased significantly above control levels under the influence of both hydrocortisone and insulin (see 4.2), neither hormone effected any $^{32}P$ incorporation into histone at this time.

Though the possibility of NHC protein contamination in our histone preparations cannot be excluded, the results indicate that the hormonal influence on phosphorylation preceded that on RNA synthesis (see 4.2).

4. 3. 3. METHYLATION :

Methylation occurs after the formation of the peptide bonds (95,97) in all histone fractions (88) except the very lysine-rich f1 (63,191,259). The biological significance of the histone methylation reaction remains to be elucidated (66,191,232,257). No time correlation has been found between histone methylation and the synthesis of RNA, DNA, histone or NHC protein (98). In experiments with phytohemaglutinin-treated lymphocytes (97), synchronized cell cultures (63,95) and in regenerating liver (191), lysine residues have been identified as the carriers of methyl groups (63,89,90) chiefly in the arginine-rich histones (63,191,258) after DNA synthesis and histone synthesis have begun to decline (191). Since this modification of the basic proteins would augment their net charge (mono- and trimethylation, see page 59) and hence increase their affinity for DNA, various authors have implicated the reaction with the compression of the chromatin prior to mitosis (63,191). The specificity of the methylating enzymes (41,92-94,260,261) for certain residues (92,93) of particular histone fractions (92), and their apparent tissue specificity (88), suggest that this alteration is inherent in the function of histones.

Nuclear methylase enzymes ('Methylase III' (260) ) have been isolated from various sources (94,96,97,259,260). These enzymes transfer the methyl group of S-adenosyl methionine (95-97) to the ε-amino groups of lysine (63,88,258) preferentially in arginine-rich histones (258,260) yielding mono- (63,89), di- (63) and tri- (90) methyl derivatives. Not all lysine residues are, however, thus affected - approximately one in eleven lysine residues of calf thymus histone f2a1, and 1 in 12 - 13 residues of f3 are methylated (67,238). A pertinent observation of Bonner et. al. (238) was that ε-N-acetyl lysine and ε-N-methyl lysine appear in a cluster of five basic amino acids in the primary DNA binding site of fraction f2a1.

Gershey et. al. (91) have detected a more pronounced methylation of lysine residues in immature erythrocytes than in the inactive mature cells. The association of methylation with the alterations in the chromosome structure preceding mitosis appear to involve these lysine residues.

Gallwitz (92), however, has identified two nuclear trans-methylases from rat thymus, one of which (IIN) preferred histone f2a1, while the other (IN) preferred histone f3 as a substrate. The major products were not methylated lysine residues, but arginine residues other than ε-N-methyl arginine. Other groups (40,41,262,263) had previously shown that in contrast to those of rat thymus, calf thymus histones contained negligible amounts of these altered residues.

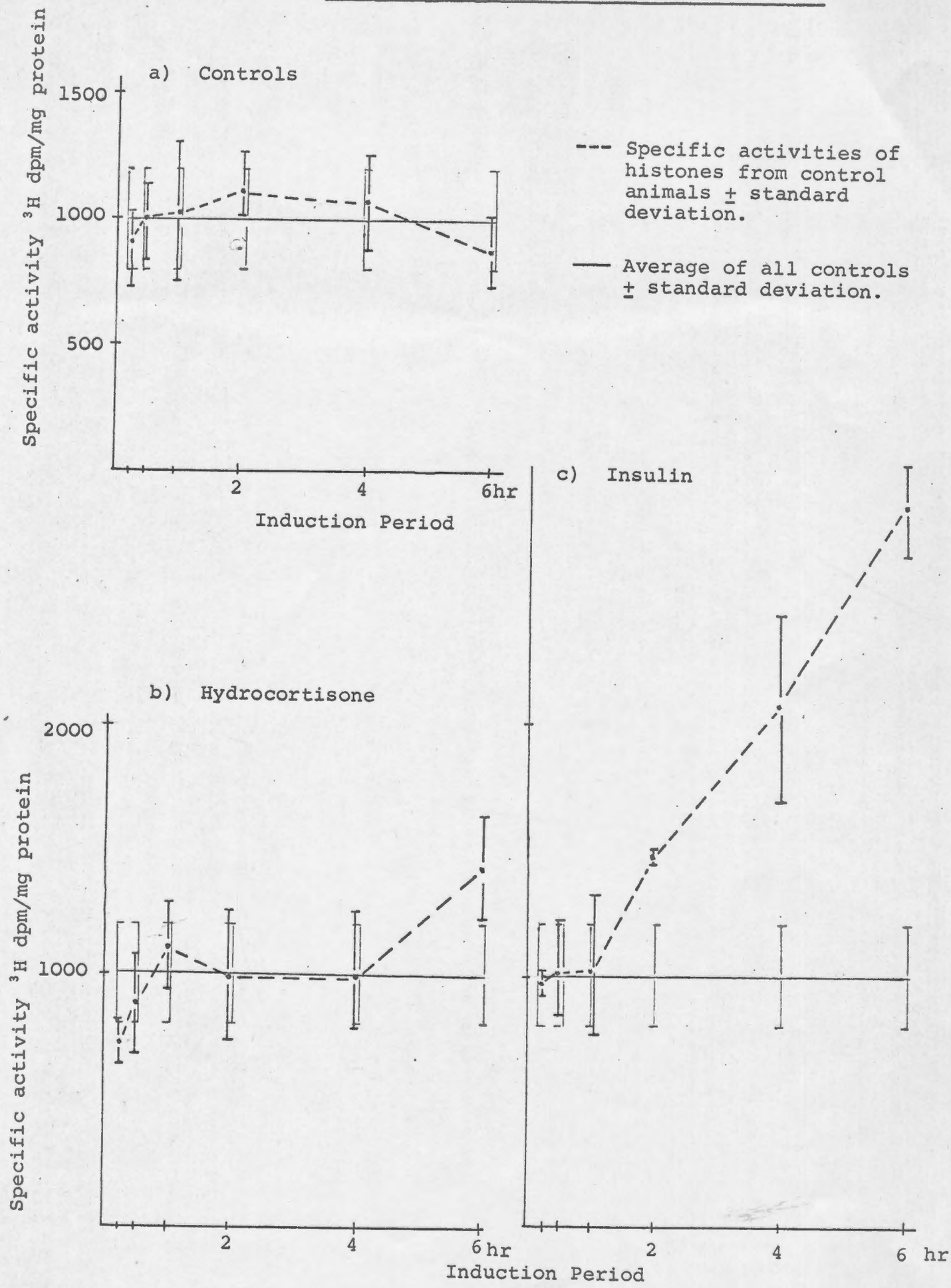
Rat liver proteins were specifically (41) modified by cytoplasmic (41,93,261) "Methylase I" (261) yielding ε-N-methyl arginine and α-N-methylguanidinomethyl arginine residues (40,88). Here the slightly lysine-rich fraction f2b was altered in advance of (63) and more than (41,262,263) the arginine-rich fractions f2a1 and f3. The activity of this enzyme was significantly diminished in estradiol 17β-treated immature rats (41). Kaye et. al. (41) have proposed that this methylation of arginine residues takes place before the histones are transported to the nucleus, hence prior to their attachment to DNA.

In addition to the above, a cytoplasmic "Methylase II" (93) transfers the methyl group to free carboxyl groups of various proteins, including histones. The enzyme catalyzing the formation of 3-methyl-histidine found in fowl erythrocytes (91) and Novikoff hepatoma (88) has not as yet been isolated.

Our tests examined histone methylation on the basis of $^{3}$H-methionine incorporation (see 3.4.). Hydrocortisone effected no variation from control values until 6 hours after its administration, whereupon a 40% increase was noted (Figure 4.3.5., Table 4.2). Since both TAT activity and RNA synthesis had already reached a maximum at this time (4.1; 4.2), the enhanced methylation here may reflect the beginnings of reactions in the chromatin not directly related to the induction of enzyme synthesis.

The injection of insulin, however, initiated a startling rise in methyl uptake within 2 hours; the increase was expanded after 4 hours to twice the control values, and at 6 hours still further to three times the control level (Figure 4.3.5., Table 4.2).

Figure 4. 3. 5. EFFECT OF HYDROCORTISONE AND INSULIN ON HISTONE METHYLATION

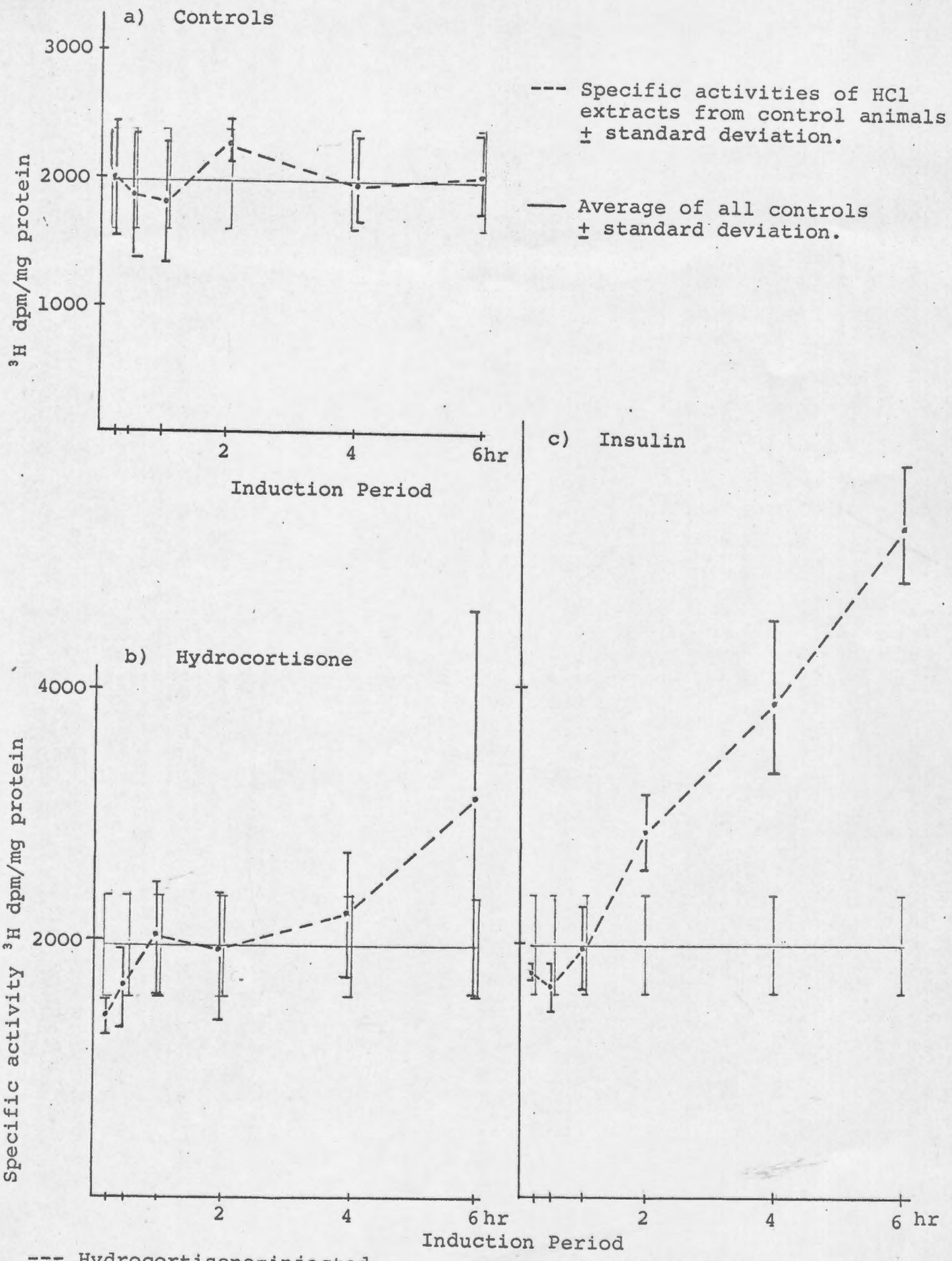



b) --- Hydrocortisone-injected.
—— Control average as above

c) --- Insulin-injected.
—— Control average as above.

50 µCi $^{3}$H-methionine/200-250 g rat were injected 15 minutes before sacrifice. Each point is the average (± standard deviation) of a minimum of 3 experiments.

Figure 4. 3. 6. EFFECT OF HYDROCORTISONE AND INSULIN ON METHYLATION OF HCl EXTRACT PROTEINS




50 µCi $^3$H-methionine/200-250 g rat were injected 15 minutes before sacrifice. Each point is the average ± standard deviation of a minimum of 3 experiments.

Reports on the methylation of all possible protein contaminants in histone preparations suggest that these are methylated to a much lesser extent (or not at all) than the histones themselves (40,88,89). Comparison of the $^{3}H$-content of our HCl extracts and histone solutions (Figure 4.3.6., Table 4.2) indicate that the electrophoretically identified contaminants in the HCl extract (Figure 3.4b) were indeed labelled.

Since low molecular weight RNA has been found in close association with the chromatin (142-146) and the methylation of tRNA (267) has been shown to increase at times of increased genetic activity (268,269), it was necessary to ensure that the content of radioactive methyl groups in our histone preparation was not due to contamination with RNA, but indeed to a genuine histone methylation.

Methylated histone samples were therefore subjected to RNase to investigate the possible presence of contaminating RNA. Such samples proved to be unaffected by this enzyme (see methods 3.3.4) indicating that the preparations were free of digestible RNA.

Trypsin digestion of methylated histone resulted in the formation of labelled peptides and amino acids (high voltage electrophoresis and TCA precipitation - section 3.3.4). Acid hydrolysis of radioactive histone was followed by adsorption of the degraded peptides and amino acids onto Dowex 50 (X4) $H^{+}$ ion exchanger. This yielded a water eluent devoid of radioactivity, and 100% recovery of the applied radioactivity in the $NH_4OH$ eluent (see 3.3.4).

The increased methylation seen after insulin administration was therefore indeed due to histone modification and not to tRNA methylation.

Burdon and Pearce (79) have furnished evidence for a methylating enzyme present in the chromatin of Krebs 2 ascites tumour cells (264) which modifies both histone and DNA. Kinetic studies implied that DNA methylation eventuated as a result of transfer of methyl groups from the histone lysyl residues (264,265).

Cyclic AMP was found to be a specific stimulator of the modification reaction (79). Chromatin itself has both cyclase and cAMP phosphodiesterase activity and the latter is inhibited by low concentrations of certain hormones (79). Considering that cyclic AMP is known to be the secondary mediator of insulin action in vivo (79,276,266) it is tempting to relate our results concerning histone methylation in response to insulin, with the reaction described above (79).

Paik and Kim (295) have compared the basic dissociation constants of ε-amino groups of lysyl residues and their methyl substituted compounds. They report the basicity scale - $N(CH_3)_2$ > - $NH_2$ > $-NH(CH_3)$ > $-(N(CH_3)_3)^+$ in increasing order. Dimethylation of such lysyl residues would therefore have an opposite effect to that of trimethylated compounds. The dimethylation could thus result in a decreased basicity and possibly affect the histone-DNA interaction in a manner similar to that of acetylation and phosphorylation.

The highest methylase activity found in rat liver is that of the nuclear 'Methylase III' (295) which specifically modifies lysyl residues (260). If this substitution results in an increase of dimethylated residues, it can be envisaged that the histone-DNA bonds are thereby weakened leading to an increased availability of the DNA for transcription.

TABLE 4. 2. INCORPORATION OF RADIOACTIVITY INTO HCl EXTRACTS AND HISTONE SOLUTIONS

Each value represents the average (± standard deviation) of a minimum of 4 animals.

Experimental animals were injected intraperitonaeally with radioactivity 15 minutes before sacrifice - 50 µCi Methionine-$^{3}H$, 80 µCi Acetic acid-1-$^{14}C$, 400 µCi $^{32}P$/200-250 g body weight.

Specific activities (dpm/mg protein) of HCl Extracts and Histone Solutions

| Induction Period | $^{32}P$ HCl Extracts | | | $^{32}P$ Histones | | |
|---|---|---|---|---|---|---|
| | Hydrocortisone | Controls | Insulin | Hydrocortisone | Controls | Insulin |
| 15 minutes | | 3681.2 ± 283.7 | 2472.8 ± 451.6 | 598.3 ± 76.3 | 841.14 ± 379.8 | 525.6 ± 181.8 |
| 30 minutes | 2895.6 ± 489.1 | 3535.1 ± 302.5 | 5626.4 ± 153.4 | 547.0 ± 29.0 | 757.2 ± 145.06 | 1224.9 ± 2.54 |
| 1 hour | 2613.8 ± 356.9 | 3911.7 ± 1041.2 | 6776.8 ± 580.7 | 605.7 ± 73.5 | 836.2 ± 204.7 | 1433.8 ± 30.6 |
| 2 hours | 3105.3 ± 251.2 | 3641.8 ± 981.2 | 3899.2 ± 1046.6 | 568.4 ± 53.4 | 798.7 ± 214.4 | 765.2 ± 106.3 |
| 4 hours | 5345.9 ± 307.2 | 4129.5 ± 768.5 | 4627.3 ± 774.3 | 733.7 ± 69.9 | 682.1 ± 136.95 | 843.2 ± 135.0 |
| 6 hours | 4615.3 ± 540.2 | 4615.3 ± 540.2 | 3858.4 ± 273.1 | 910.7 ± 57.14 | 895.75 ± 145.02 | 1007.6 ± 92.1 |
| | | Average of all controls : 3846.7 ± 734.4 | | | Average of all controls : 796.8 ± 213.1 | |

TABLE 4.2. /cont'd....

Specific activities (dpm/mg protein) of HCl Extracts and Histone Solutions

| Induction Period | $^{14}C$ HCl Extracts | | | $^{14}C$ Histones | | |
|---|---|---|---|---|---|---|
| | Hydrocortisone | Controls | Insulin | Hydrocortisone | Controls | Insulin |
| 15 minutes | 395.02 ± 111.5 | 567.35 ± 132.3 | 596.15 ± 140.5 | 317.7 ± 116.9 | 378.04 ± 117.2 | 513.6 ± 73.4 |
| 30 minutes | 556.1 ± 132.1 | 466.2 ± 124.5 | 830.7 ± 190.1 | 448.6 ± 108.6 | 343.2 ± 104.6 | 606.89 ± 157.8 |
| 1 hour | 554.2 ± 59.1 | 462.5 ± 119.8 | 511.5 ± 199.1 | 438.6 ± 46.1 | 407.2 ± 101.5 | 539.6 ± 173.0 |
| 2 hours | 625.78 ± 13.4 | 516.1 ± 77.4 | 644.5 ± 56.8 | 357.3 ± 17.7 | 362.2 ± 91.3 | 530.4 ± 46.1 |
| 4 hours | 424.05 ± 52.1 | 360.4 ± 67.0 | 676.1 ± 214.1 | 308.65 ± 25.8 | 299.5 ± 93.9 | 480.2 ± 188.4 |
| 6 hours | 438.5 ± 65.3 | 469.6 ± 109.5 | 775.15 ± 94.9 | 341.2 ± 7.84 | 371.7 ± 98.1 | 675.75 ± 100.0 |
| | | Average of all controls : 464.7 ± 121.5 | | | Average of all controls : 358.6 ± 102.8 | |
| | $^{3}H$ HCl Extracts | | | $^{3}H$ Histones | | |
| 15 minutes | 1404.95 ± 134.0 | 2003.4 ± 428.2 | 1781.6 ± 44.2 | 740.2 ± 88.2 | 904.2 ± 187.4 | 978.05 ± 26.6 |
| 30 minutes | 1676.9 ± 278.3 | 1876.3 ± 502.1 | 1658.3 ± 187.8 | 891.3 ± 194.9 | 1002.4 ± 168.9 | 1029.1 ± 203.7 |
| 1 hour | 2056.75 ± 476.8 | 1801.9 ± 469.3 | 1974.4 ± 316.6 | 1122.5 ± 180.0 | 1037.5 ± 273.5 | 1074.8 ± 156.7 |
| 2 hours | 1919.3 ± 569.8 | 2296.9 ± 163.05 | 2882.6 ± 287.4 | 1006.25 ± 261.4 | 1165.3 ± 148.9 | 1466.1 ± 16.8 |
| 4 hours | 2209.9 ± 488.6 | 1980.5 ± 321.4 | 3948.3 ± 606.6 | 1090.5 ± 177.5 | 1083.7 ± 205.3 | 2175.25 ± 477.12 |
| 6 hours | 3107.8 ± 1507.9 | 2048.7 ± 320.4 | 5298.25 ± 478.7 | 1437.1 ± 205.0 | 879.5 ± 130.3 | 2881.0 ± 231.9 |
| | | Average of all controls : 1984.6 ± 398.0 | | | Average of all controls : 1008.2 ± 198.9 | |

## 5. DISCUSSION AND CONCLUSIONS :

Various theories on the subject of the repression and depression of the genome have been considered in Section 1. The involvement of histones seems implicit although their exact role remains uncertain. Other components present in the chromatin appear to influence the nature of DNA-histone complexes thereby conferring specificity to the suppressive functions of these proteins. Modifications of histone amino acid side chains by acetylation, phosphorylation and methylation seem to be immanent processes in the regulation of their function. Varying structural alterations as response to the activation of cellular activity have been reported. Active chromatins have been noted to contain more acetylated (65,187,190,223) phosphorylated (37,38,65,190,270,271) and methylated (91,289) arginine-rich histones (65,91,190,223) than their inactive forms. Some workers deny this (272,273). Phytohemaglutinin-stimulated lymphocytes exhibit an early increase in RNA synthesis - again, both positive (34,37,188,189,247,249) and negative (274) correlations with histone modifications have been described. A limited number of reports (123) refute such structural variations in the course of liver regeneration (63,86, 102,189,191,241,242,255,275) after partial hepatectomy. With regard to these responses to either steroid (36,40,41,188,222,224) or polypeptide (36,68,76-78,251,254,274) hormonal influence, few authors agree.

Notwithstanding, the consequence of gene activation is transcription. Enhanced template activity of the chromatin (12-16) and increased activity of endogenous RNA polymerases in target cells, are well established results of hormonal treatment (2,6-8,32,110). The accompanying synthesis of RNA includes all types (162,163) - the initial precursor molecules, ribosomal (171-174), transfer (164,177) and DNA-like (173,174,219,221) attended by their matured "degradation products" (173-175). Partial hepatectomy (28,163) and steroid hormone administration (277,278) appear to cause an alteration in the distribution of RNA species; some workers have reported an increase in GC-rich species (28) (ribosomal (27) ), others a multiplication of the AU-rich (DNA-like (27) ) species (163).

The ensueing protein synthesis is dependent on the synthesis and transport to the cytoplasm of rRNA, mRNA and tRNA. One point on which all researchers agree, is that RNA synthesis precedes hormone-induced protein synthesis. The present time course studies on the hepatic responses to hydrocortisone and insulin substantiate this, although the preceding events appear to differ with the two hormones.

Accepting that our conditions of measurement had their limitations (see 4.3) we found that the effect of hydrocortisone encompassed but trivial variations in total histone modification. $^{14}$C-Acetate and $^{32}$P incorporation showed minor changes early in the induction period, while $^{3}$H-methionine incorporation remained at control levels until 6 hours after hormone administration, at which time there was a slight rise in methylation.

RNA synthesis, however, was substantially enhanced. That of high molecular weight species reached a maximum within 4 hours. The low molecular weight species reached their maximum 6 hours after hormone injection (see 4.2) although the 10S region, which would contain mRNA (164,173,221) already showed a definite increase after 2 hours. Concomitant with these events was the intensification of TAT activity to twice control values 4 hours, and four times control values 6 hours after the administration of hydrocortisone.

The influence of insulin on TAT, in comparison with that of hydrocortisone, was slightly delayed - the extracts from injected animals retained control activities until the 4 hour period to rise between 4 and 6 hours to a five-fold increase. The preceding histone modifications were, in contrast to those under hydrocortisone, conspicuously augmented by the presence of insulin. Our results confirm those of authors who have implied greater acetate and phosphate uptake in advance of RNA synthesis (36,65,188,190). Acetylation was shown to expand 30 minutes after hormone injection, and subside to control values for the remainder of the experimental period. Phosphorylation (whether it involved histone and/or NHC protein (see 4.3.2) proceeded by a similar pattern. Our observations regarding methylation were unexpected. The reports of Burdon et. al. (79) on stimulation of chromatin methylase activity by cyclic AMP and the fact that cyclic AMP is a secondary mediator in induction by insulin (276) could relate to the means by which this reaction was executed.

Turner and Hancock (289) have reported a higher methylase activity in fetal liver extracts (i.e. with high cellular activity) than in adult liver extracts. Possibly the transmethylation reaction described by Pearce et. al. (264,265) by which methyl groups are transferred from histone to DNA would result in a release of protein from the DNA-histone complex. Since cyclic AMP (which specifically activates this reaction (79)) is a secondary mediator of insulin action (276), our results could be a reflection of the first step in this transmethylation, resulting in increased transcription.

If the increased methylation involved mostly dimethylation of ε-amino groups of lysyl residues, it is possible that the decreased basicity resulting from such modification (295) would cause a weakening of the DNA-histone bonds and hence an increased availability of the DNA for transcription.

Alternatively, if the increased methylation involved mono- and/or trimethylation of such residues, the result would be a net enhancement-of-charge (63,295) and possibly a consolidation of the DNA-histone interaction (46). Insulin is known to repress the activity of certain hepatic enzymes (300-302). The enhanced methylation in this case could be an expression of a shutting-down mechanism for the purpose of repression. Therefore, although TAT activity be increased under the influence of insulin, other enzymes may well be repressed.

Histone modification appeared to be an equivocal event in the steroid-induced amplification of RNA and protein synthesis. If histones be involved, their direct interaction with hormone (16,151,158) and/or regulatory proteins (12-14,279,280), with the RNA polymerase enzyme (50,51,57,281) or their modified associations with NHC proteins (35,105,115,134,140,247) are alternative mechanisms by which they may mediate induction by the steroid hormone. Translational level control (282,283) has also been described. On the other hand, the involvement of these structural modifications of histone in insulin induction is ratified by their distinctive response to this hormone.

In this investigation two different hormones have induced the same enzyme activity. The patterns of histone modification preceding and accompanying RNA and enzyme synthesis were, however, distinctly different. TAT activity is but one indicator of increased transcriptional and translational activity in response to the two hormones. Each induces a number of enzymes. Therefore what may appear a qualitative difference in the modification of histones, could well be a quantitative difference in the number of enzymes induced by either insulin or hydrocortisone.

Another explanation cannot be neglected, namely, that the two hormones trigger transcription in different ways. It can be envisaged that the gene for TAT exists either as a single copy or as several copies physically attached to different hormone receptors. Each of these receptors, on combination with the inducer, could initiate a different series of events leading to the same result, i.e. the exposure of the histone-covered gene to the DNA-dependent RNA polymerase.

SUMMARY

The in vivo effects of hydrocortisone and insulin on histone modification (acetylation, phosphorylation and methylation), RNA and enzyme synthesis in rat liver have been investigated.

1. Both hormones were found to induce tyrosine transaminase activity to between 4 and 5 times control values within 6 hours of their administration.

2. RNA synthesis, involving all species, was increased within 2 hours, the high molecular weight species reaching a maximum at 4 hours, and the lower molecular weight species reaching their maximum at 6 hours.

3. Hydrocortisone effected minor alterations in histone modification - $^{14}$C-acetate and $^{32}$P incorporation showed slight changes early in the induction period, while $^{3}$H-methionine incorporation remained at control levels until 6 hours after hormone administration at which stage there was a slight rise in methylation.

4. Insulin injection resulted in significant increases of all three forms of histone modification. Acetylation and phosphorylation increased in advance of the enhanced RNA synthesis, while the rise in methylation continued almost linearly from 2 hours to 6 hours after administration of the polypeptide hormone.

The different means by which hydrocortisone and insulin accomplish their inductive effects is briefly discussed.

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